ADVANCES IN ENZYMOLOGY

AND RELATED SUBJECTS OF BIOCHEMISTRY

Volume IV

CONTRIBUTORS TO VOLUME IV

WALLACE R. BRODE, *Department of Chemistry, The Ohio State University, Columbus, Ohio*

C. R. DAWSON, *Department of Chemistry, Columbia University, New York, N. Y.*

ADDISON GULICK, *Department of Biochemistry, University of Missouri, Columbia, Mo.*

ROBERT M. HERBST, *Knollwood Road, Short Hills, N. J.*

ROLLIN D. HOTCHKISS, *Lieutenant-Commander, Hospital Corps, USNR, Rockefeller Institute for Medical Research, New York, N. Y.*

H. JENSEN, *Research Laboratories, The Upjohn Co., Kalamazoo, Mich.*

J. M. NELSON, *Department of Chemistry, Columbia University, New York, N. Y.*

WILLIAM WARD PIGMAN, *U. S. Department of Commerce, National Bureau of Standards, Washington, D. C.*

V. R. POTTER, *McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wis.*

LEON E. TENENBAUM, *Research Laboratories, The Upjohn Co., Kalamazoo, Mich.*

ADVANCES IN ENZYMOLOGY

AND RELATED SUBJECTS OF BIOCHEMISTRY

Edited by

F. F. NORD
Fordham University, New York, N. Y.

C. H. WERKMAN
Iowa State College, Ames, Iowa

VOLUME IV

CONTENTS

THE CHEMICAL FORMULATION OF GENE STRUCTURE AND GENE ACTION

By

ADDISON GULICK

Columbia, Missouri

CONTENTS

I. Introduction

For the past four decades, since the rediscovery of Mendel's laws, the science of genetics has been progressing consistently from the status of a group of formal abstract propositions toward a more concrete science dealing with specialized strings of material particles possessed of certain outstanding attributes. These particles—genes—belong to the order of magnitude of megamolecules, and to the extent that the chemist has undertaken the study of megamolecules he can claim them as within his field. Thus, chemical genetics is a subject capable of very broad definitions; a chemical geneticist is not a scientist in charge of a particular subdivision of genetics, but is, much of the time, one who differs from other geneticists merely in the mental tools he brings to almost any genetic problem.

Is there any chemical pattern that all genes possess in common? Wherein is found the individuality of each particular gene, without which it could

1

not serve as a distinctive determinant of some particular characteristic or characteristics? When a gene acts, what is happening, chemically speaking? All such questions come into the field of chemical genetics.

II. Protein Molecules

Our concepts of the constitution of genes are closely dependent upon the structural picture we make of the protein molecule. Here, the latest advances have resulted from several modes of attack: x-ray diffraction studies, centrifugal and other methods for determining molecular size, studies of enzyme hydrolysis and the linkages involved, increasingly precise analysis of amino acid content and, most recently of all, electron microscope shadow pictures of the particles of dissolved homogeneous proteins.

Several lines of evidence tend to confirm the orthodox opinion, coming from Emil Fischer and Franz Hofmeister, that proteins consist fundamentally of polypeptide chains. In the fibrous proteins (keratin, myosin, white connective fiber, silk, etc.) the entire molecule may be a single, exaggeratedly long peptide chain. Protamines are similar strings but shorter, some 15 to 33 amino acid groups in a linear series constituting one molecule.

The peptide linkage by which the amino acids are joined seems even more certain today than it has seemed in the past, since protein-splitting enzymes are being identified more and more universally as splitters of the peptide linkage. It seems probable today that there is no other kind of proteolytic action, hence that the fundamental structural bonds are all of this type.

But not all protein molecules consist of a single, straight, linear fiber; and these less fibrous molecules have been the object of considerable speculation. Are they bundles of parallel fibers, or are they folded fibers, or a flat fabric, or a cage (5, 6, 45, 82, 203–214)? It seems clear that structurally and genetically they stand very close to the fiber proteins, so that only refined physical tests can be trusted to show that they are different. Very gentle physical forces are often sufficient to change the compact type of molecule (sometimes less accurately called "globular") into a film or even a peptide filament. Also, both filamentous and massive proteins are prone to follow the "Svedberg number series" and the "Bergmann ratios," upon which some comment is needed.

The Svedberg series expresses the observation that numerous proteins fall in the series having molecular weights of 16,000 to 17,000, or of 32,000 to 34,000, and multiples, roughly $(2^m \times 3^n) \times$ *ca.* 33,000. The lowest

number in this series (*ca.* 16,000) is found to represent the weight of a protein having not far from 144 amino acids in its unit molecule, the range of variation being due to differences possible in the average molecular weights of the amino acids represented in different proteins. Similarly *ca.* 33,000 represents 288 residues, and the higher members of the series stand for an amino acid content of 2, 3, 4, 6, 8, 12, etc., times the fundamental number, 288.

The Bergmann ratios express the finding that, in numerous proteins, the various amino acids are represented in fractional ratios such as $1/2$, $1/4$, $1/6$, $1/8$, $1/12$, $1/18$, $1/24$, $1/36$, $1/72$, etc., that can be summarized as $(2^m \times 3^n)/288$, or figures easily derived from this series. Simple variants such as $5/16$ (*viz.*, $1/4 + 1/16$) also can occur. Bergmann (15) explained his series by supposing that the different residues occur repeatedly at regular intervals along the chain; for example, one amino acid occupying every fourth position, another every sixth, another every eighth, etc.

Studies made on certain particularly favorable nonfibrous proteins have brought at least a partial confirmation of the Svedberg and Bergmann numbers, although we find indications that, before they can be considered a universal feature of proteins, at least some reinterpretations of special cases will be needed. The especially favorable insulin molecule gives the apparent amino acid ratios in Table I.

TABLE I

COMPOSITION OF INSULIN-ZINC*

Component	Per cent by wt.	mM. per 100 g. protein	mM. per 100 g. protein (calcd.)	Fractional frequency	Residues per 288
Zinc	0.52	8	9		(3)
Lysine	2.26	15	17	1/48?	6?
Arginine	3.22	18	17	1/48	6
Histidine	8.00	52	51	1/16	18
Tyrosine	12.20	67	68	1/12	24
Cystine (as cysteine)	12.60	104	102	1/8	36
Ammonia	1.65	97	102	...	36?
Glutamic acid	30.00	204	204	1/4	72
Leucine	30.00	229	204	1/4?	72?
Phenylalanine	0.0?	...	...	...	0?
Proline	0.0?	...	...	...	0?

* According to du Vigneaud (196). Calculations by du Vigneaud are based on Svedberg's determination of the molecular weight, 35,100. Miller and Andersson (131) believe it should be 46,000, which may mean a particle with 3×144 amino acids.

There has been much discussion of the question whether the insulin molecule has the structure of a cage assembled out of a series of membranous polypeptide aggregates, such as the cyclol pattern for which Wrinch (207, 208) has offered persuasive geometrical arguments, or whether it is built according to some quite different system out of compacted polypeptide chains. Fourier diagrams of the fields of electron concentration in this molecule seem to indicate that there are 18 foci of concentration located within the essentially octahedral space of the molecule (45). This number equals the sum of the edges and corners found in an octahedron. However, no published study has yet stated the geometrical distribution of the foci. If these 18 foci are the signatures of 18 compacted polypeptide chains of 16 amino acids each, to make up the total content of 288, then the Bergmann ratios find a simple interpretation as an outcome of a fair degree of rhythmic similarity in these several polypeptide chains. Whether built thus in moderate-sized block units, or formed of membranous units assembled into a cage, it appears that the assembled molecule of Svedberg size must have an arrangement which stows the hydrophobe groupings largely inside and exposes hydrophil groupings at the surface. This is shown by the solubility of the intact molecule and relative insolubility of the material when disarranged or slightly hydrolyzed (209, 215).

The ease with which many proteins ravel out from the compact molecule into a fibrous form leads to the widespread opinion that the bonds responsible for the compacting process are not major valency bonds, but represent electrical attractions that polar regions of the amino acid residues exert upon one another. The resulting shapes are not random, however, and each crystallizable native protein must have a very exact pattern according to which its polypeptide chain or chains are coiled. Possibly there may be a similarity to the "cyclol" fabric, as Wrinch has shown that very nearly the same cyclol geometry could be applied by employing hydrogen bonds to draw the amino acid residues into flat hexagons (214).

III. Autosynthetic Molecules

Troland (195) and later Koltzoff (95, 96) foresaw that the key to biological reproduction must necessarily be some sort of self-reproducing protein molecule. Such a molecule would be definable as an enzyme presiding over the processes of its own synthesis. It would also constitute a unit of heredity. In short, it would be the sort of a thing that a gene is. But the genes of most plants and animals are impossible to obtain as yet in quantities which are ponderable and analyzable, if, indeed, procurable in sufficient bulk even to be separately visible under the microscope. The first substance successfully isolated that fitted Koltzoff's specifications was the tobacco mosaic disease virus, isolated as a compound by Vinson (197), and in pure form by Stanley (181–188) out of the sap of diseased plants. This discovery led the way to the successful isolation of a con-

siderable list of viruses, from diseased tissues of both plants and animals, and on this basis some generalizations have become possible (72, 125, 126).

All these viruses are nucleoproteins, and hence are comparable to the principal material in the chromosomes of higher organisms. But the strongest resemblance (at least of the tobacco virus substance) is to the so-called nucleoproteins of cytoplasm rather than to true nuclear material: first, because their nucleic acid is not built on the framework of desoxyribose as we find the major nucleic acids of all animal nuclei and most plant nuclei to be constituted, but on the framework of a more typical pentose sugar, presumably ribose, as is usual in most of the cytoplasmic nucleic acids of both plants and animals; and secondly, because the protein constituent of viruses has the dimensions and weight of a giant molecule, not
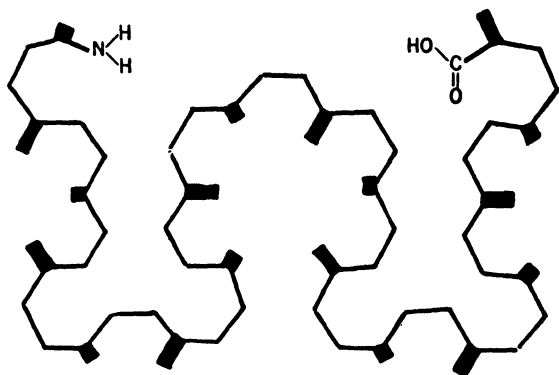
Fig. 1.—Polypeptide loops according to the cyclol pattern
(Wrinch, 206–209).

that of a protamine or histone, and is formed of an assemblage of amino acids comparable to those found in ordinary proteins and not especially provided with the highly basic amino acid moieties, such as are found in chromosomes. The last point leads to the further difference that a relatively high percentage of protein is found combined with a relatively low percentage of nucleic acid. Fish-sperm nucleoprotein is roughly two-thirds nucleic acid and one-third protamine, in contrast to tobacco virus, which contains 6% nucleic acid and 94% protein. Examples could be chosen, however, which give a much less striking contrast.

The structure of viruses is crystalline rather than biologically cellular. The active tobacco virus is revealed by electron microscope shadow pictures (187) as minute rods about 2800 Å long and about 150 Å in diameter. These may be built into hexagonal crystals, or into long filaments which are probably only imperfectly crystalline. Stanley

finds that no unit smaller than these rods has evinced active virus potency. They represent a molecular weight of about 40,000,000; and, since their size seems to be constant as long as the virus efficacy is unimpaired, this is looked upon as the molecular weight of the nucleoprotein megamolecule.

X-ray diffraction studies (17) bring out an intricate and highly stable structure within the individual rods; and indeed it is possible from this standpoint that the unit of crystal repetition is a small fraction of the gross size of the megamolecule, having the form of hexagonal prisms 68 Å tall and 87 Å across, giving a total volume of about 450,000 cu. Å and, at sp. gr. 1.34, a contained molecular weight of 370,000. There is also an important transverse spacing at 44 Å, and a longitudinal demarkation at 22 Å; and further diffraction spots seem to indicate 11 Å spacings in all three dimensions, as well as finer spacings between amino acid residues, and between even smaller atomic groups. These complex closer spacings have not yet been successfully interpreted.
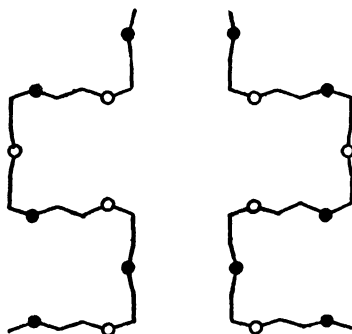
Fig. 2.—Polypeptide loops according to the alpha-keratin pattern (Astbury and Bell, 6).

It is of interest that x-ray diffraction shows a spacing of 11 Å in all three directions, as if the peptides were stowed in cubes of that dimension. There is no known block of amino acids the right size to fit such a cube, unless it be a loop of a heavily serpentine chain. Figures 1 and 2 illustrate patterns for looping that conform to the concepts of Wrinch (207–214) and of Astbury and Bell (6). Neurath (145) has argued that Wrinch's cyclol (208) fabric fails to give necessary space to the amino acid residues; but this difficulty is less serious with small loops held together laterally only by accessory bonds. (See 214.)

Quantitative chemical analyses (94, 159) indicate a minimum molecule corresponding to the Svedberg series (Table II).

Despite its other differences, the nucleic acid portion of the tobacco virus resembles that of chromosomes in having the form of very highly polymerized fibers in which the nucleotide units lie crosswise, closely packed,

like a stack of coins. Virus nucleic acid is credited (41, 111) with a length of 3000 Å and a molecular weight of 300,000 corresponding to a content of about 800 nucleotide units, or about 200 tetranucleotides. This rather inflexible structure equals or exceeds the standard virus particle in length, so that it must be stowed longitudinally, possibly even in a gentle spiral.

TABLE II

AMINO ACIDS IN TOBACCO MOSAIC VIRUS*

Constituent	% as amino acid	mM. per 100 g.	Calculated min. mol.	Svedberg units	Frequency per min. Sved. unit
Alanine	2.4	...	...	....	...
Amide nitrogen	1.9	112˙	...	....	...
Arginine	9.0	52	1811	16,300 or 32,600	9
Aspartic acid	2.6+	...	...	....	.. ⅜
Cysteine	0.7	5.8	16310	16,310 or 32,620	1
Glutamic acid	5.3+	...	...	....	.. •
Glycine	0.0	...	...	....	...
Histidine	0.0	...	...	....	...
Leucine	6.1+	...	...	....	...
Lysine	0.0	...	...	....	...
Methionine	0.0	...	...	....	...
Nucleic acid (as nucleotides)	5.8	18.5	5090	15,270 or 30,540	3
Phenylalanine	6.0	36.4	2585	15,510 or 31,020	6
Proline	4.6	40.0	2344	16,510? 33,020?	7?
Serine	6.4	61	...	17,061? 34,122?	11?
Threonine	5.3	44.5	2134	17,070 or 34,140	8
Tryptophane	4.5	22.1	4286	17,143 or 34,286	4
Tyrosine	3.9	21.5	4494	17,574 or 35,148	4
Valine	3.9+	...	...	....	...

* Data from Ross (159). The molecular weight columns of this table are calculated in terms of nucleic-acid-free protein.

The spacing between nucleotides is very close indeed, probably about as close as the 3.34 Å observed in chromosomal nucleic acids. About nine filaments of these polymeric nucleic acids must be required to supply the 7200 phosphorus atoms that are found in each virus particle.

In résumé of the tobacco virus studies, we find, then, that self-reduplicating virus substance is wafer-like rather than fibrous, composed of peptide units sufficiently small to be completely penetrated by the electric and electromagnetic fields which govern crystal formation. Since these lowest units, about 11 Å cubes or pairs of cubes of this size, contain surely not

over eighteen amino acids each, different ones must possess different assortments of amino acids. These smallest peptide units are associated in wafer-shaped units of next higher order, measuring 44 Å across, and probably 22 Å in thickness. It then appears most probable that during self-reduplication, formation of an assemblage of units of the lowest order leads up to the construction of a complete wafer, and that at least in major part the virus consists of repeats of similar wafers, plus the strands of nucleic acid to which they are joined.

The tobacco mosaic virus represents apparently one of the largest types of virus particle that can still be regarded from the chemically unitary standpoint. The next larger category of virus particles (vaccinia, etc.) seems to be intermediate between chemical viruses and the smallest true cellular organism (9, 188; see also 160). Our interest turns then in the other direction toward more minute viruses. Bourdillon (24) investigated two such materials. The larger of them, the virus of mouse encephalomyelitis, shows a diameter in the range of 150 Å as tested by the diffusion method. While this method is incomplete without fuller data on the shapes of the particles, it offers a first approximation to a size of a molecular weight of perhaps 2,000,000, one-twentieth the size of the tobacco virus particle. The next example, influenza virus, is complicated by its propensity to ride attached to a large, indifferent particle, probably of protein. When not so attached, Bourdillon determines for it an approximate diameter of 60 Å. Chambers and Henle (36, 37), working with an influenza virus but by centrifugation, determined a diameter of 110 Å. Such dimensions correspond to molecular weights in the ranges of 90,000 to 560,000, the equivalent of about 3 to 16 Svedberg units or albumin molecules. We may well look upon these last findings as representing an approach to the minimum limit of size for self-propagating particles.

IV. The Autosynthetic Process

Biological autosynthesis, as has already been remarked, is observed exclusively where there are special enzymatic proteins functioning in close union with nucleic acid. Synthesis of both these ingredients is involved, but the two syntheses need not be simultaneous. Chromosomes entering a reproductive cycle first show a heavy increase of nucleic acid, and only afterward is there the beginning evidence of the chain of genes becoming reduplicated (33, 46, 65, 117, 133, 167–169).

Since the nucleic acid is largely nonspecific, varying little from organism to organism, the chief interest attaches itself to the protein member of the pair, and to the problem of its duplication. Like viruses and like all pro-

teins, the valency linkages undoubtedly are largely, perhaps entirely, peptide bondings, with the form

$$-\overset{\displaystyle \parallel}{\underset{\displaystyle O}{C}}-\overset{\displaystyle H}{N}- \quad \text{or} \quad -C\!=\!\underset{\displaystyle OH}{N}-$$

in long series, between amino acids that belong exclusively to the *levo*-series, as classified by the rotation of the crucial carbon. With *d-* and *l-* understood in this way, no amino acid of the *d*-series is physiological. The correct placing of a new series of amino acids to duplicate that existing in the enzymatic pattern protein must take place under the influence of short-range electrochemical forces, and must be comparable kinetically to the building of repeated new layers on the surface of a crystal. This necessitates a waferlike pattern protein, that will have all its crucial constituents located on a working surface (75). The elements of the daughter layer will assemble themselves on this surface with the same absolute orientation as their pattern, that is, back to front against it, as in the case of a crystal layer (50, 75). If it were front to front we should have to anticipate a serious disturbance of the stereoisomerism, as the only way to obtain a perfect correspondence of parts on that basis would be by bringing in the *d*-series of amino acids.

As the formation of peptide linkages between amino acids is a slightly endothermic reaction, we cannot expect this synthesis to occur except under conditions that will provide the energy. It seems probable that the group entering the synthesis is not free amino acid, but some closely related substance with a higher energy content, such as possibly the corresponding amino aldehyde (110). Such a compound could be brought into position and then undergo oxidative dehydrogenation (exothermic) to establish the necessary peptide linkage. Delbrück (50) has expanded this thought by proposing that, under the right crystallographic (catalytic) surroundings, the difficult two-step oxidative removal of hydrogen becomes easy, as the first half of the oxidation would lead to a possible condition of resonance between the preformed peptide linkage in the pattern and the nascent peptide linkage in the daughter layer of protein, a condition that would diminish the activation energy requirement of the reaction. He points out the presumption that such a reaction would be highly specific for accurate autosynthesis because it would be crystallographically conditioned.

If this hypothesis is correct, then amino acids obtained by hydrolysis of previous protein material would have to surrender oxygen to some substance undergoing oxidation before they become fit material for resynthesis. We have seen nothing impossible in such a cycle.

It is also necessary to allow for synthesis of large portions of the protein molecule out of carbohydrate plus ammonium salts or urea. The model for this is the synthesis of hippuric acid (benzoylglycine) from carbohydrate, urea, and benzoic acid. Although the hippuric synthesis seems well authenticated in the mammalian kidney, we have little information about the steps involved.

Recent studies of polysaccharide synthesis (43, 73) and allied topics emphasize the importance of phosphoric acid compounds as necessary intermediaries. The energy contained in a phosphoric organic bond is high enough so that a synthesis by replacing the acid with a carbon compound may often be accomplished without the addition of energy from outside. The removed phosphoric acid (H_3PO_4) must be provided with a new resting place; and one such possible haven (as shown by the physiology of muscle contraction) is by attachment to nucleic acid to form polyphosphates. Obviously chromosomes and viruses contain the materials for such a sequence, and one must inevitably consider whether there is not a phosphoric machinery for synthesis in the chromosomes and viruses, a mechanism in which the nucleotides are but one member. If there is, then the road from alimentary amino acid to protein would be initiated via some form of phosphoamino acid rather than by way of the cognate aldehyde. There is no need for the energy to be provided in both ways. Or if the immediate material is the aldehyde, its formation would not improbably be by way of the phospho acid (*cf.* Kalckar, 86).

When, as in the typical case of plants, there are no preformed alimentary amino acids, it becomes highly probable that phosphorylated derivatives of carbohydrates are the immediate building material both for polysaccharides and for proteins, supplemented in the latter case by some source for amino groups.

An interesting approach to the problem of protein synthesis is presented by Janssen (85). He makes a reinterpretation of the Bergmann ratios, and concludes that the real series is not an homogenous sequence factorable into $2^n \times 3^m$, but rather a double sequence, the major series of amino acids running 8×16, to which is added the postscript of a dissimilar 1×16, making a total of $128 + 16 = 144$ amino acids in the fundamental unit plus its supplement. He looks to the polymerized polynucleotide aided by adjoining protamine or histone groupings to supply an appropriate synthetic machine and mould for the segments of protein polypeptide. He proposes that glucose molecules should be packed in close formation, one beside each nucleotide, and that every alternate glucose should receive an amino group on its alpha (or No. 2) carbon, provided out of the guanidine groupings at hand. A wave of oxidation, reduction, and condensation is then supposed to produce a polypeptide chain, each amino acid of which is formed in the space occupied by two glucose units. Each amino acid is supposed to

obtain its carbon atoms from its two antecedent glucoses, except that those having three carbons or less would depend on only one glucose. In the process of linking 16 amino acids one to the other, they must draw together into considerably less space than had been taken by 32 glucose molecules. Through this shrinkage, the polypeptide must become pulled out of the sockets of its mould. The length of the strings, 16 amino acids each, is supposed to be determined by the regular occurrence at these intervals of bulky aromatic residues, such as tyrosine, that come in the way of a successful linkage. As eight such peptide chains draw together into a group, they are supposed to clear the way for a similarly engineered 16-unit sample with histone or protamine affinities, modelled by a protein unit of the nucleoprotein. Therewith the mould becomes ripe for replenishment with the needed —NH_2, —SH, etc., and for refilling with glucose. Thus the cycle is supposed to be repeated, producing each time a major block of 128 amino acids, followed by a minor block containing 16. Janssen's work is to be seen as a suggestive reanalysis of the complex numerical series of residues that the protein chemists have reported, coupled with a clever theoretical extrapolation considerably beyond the zone that is as yet factually verifiable. Hypotheses built in this way are at best liable to the need of heavy revision as the facts come in; yet, understood for what they are, they should be fertile sources of scientific concepts and stimuli to crucial experimentation. One correction to Janssen's hypothesis is already on hand, viz., enzyme experiments indicate that aromatic amino acids are not located exclusively at the basic end of protein amino acid chains.

It is particularly interesting that Janssen envisages the systematic production of a protein that is not a reduplication of a pattern pre-existing in the synthetic machine, but is predetermined by a largely nonprotein (nucleic acid) mould. Some such synthesis of a type of material not represented in the substance of the synthetic machinery must occur during gene action when the gene generates a nongenic active principle that it discharges into the cell.

Recent studies with radioactive isotope nitrogen (158) have demonstrated that the proteins in the living cell are constantly undergoing exchanges of their amino acid residues. Thus, even after it is completely synthesized, the protein molecule does not maintain its absolute identity of material, but merely a steady state with a like configuration of like materials.

V. Chromatin Chemistry

It is to be supposed that the residual materials in the chromosomes, the matrix in which the genes are located, are among the first, most immediate derivatives of the genes; and so it may be hoped that these materials reflect the nature of the genes to some extent. Chromosome chemistry is studied by microchemical staining techniques such as the Feulgen stain, by ultraviolet absorption spectra, by polariscopic methods, by x-ray dif-

fraction, by digestibility to specific enzymes, and by analyzing nuclear material microscopically after collecting purified specimens. It will be worth while to enumerate the ways in which purified nuclear material has been obtained by various investigators.

Miescher (130) previous to 1871 effected a digestion of pus cells with pepsin, or broke them down with chloroform water, obtaining an undissolved residue of nuclei. Soon afterward (1874) he reported on purification of the nuclei of salmon spermatozoa by treatment with distilled water, which partially liquefied the cytoplasmic structures and rendered the nuclei separable in the centrifuge. After that, for many years, fish spermatozoa were a favorite material for such studies, because of this relatively simple separation.

Behrens (13) devised a procedure for grinding a dehydrated tissue to an impalpable powder and centrifuging out the nuclear fraction from suspension in mixtures of benzene with chloroform or carbon tetrachloride, relying on the especially high specific gravity of chromosomal substance to bring it down from a liquid mixture that will float everything else. This is a laborious method, but very widely applicable; Behrens finds it possible for the quality of the product to be high enough so that it is at least to some degree usable for tests of enzyme content.

Quick freezing, then thawing to 30° C., disintegrates all but the nucleus of the red blood corpuscles of birds and cold-blooded vertebrates (201). Miyake (135) has found this a useful process. Yakusizi (219) has treated nucleated corpuscles with slightly hypertonic sodium or ammonium chloride to which saponin has been added, obtaining in this way a colorless suspension of the separated nuclei.

Treatment with dilute citric acid will soften and liquefy the cytoplasm of many tissues to the point at which the nuclei will slip free and can be collected by centrifugation (44). This procedure has been used by Stoneburg (189) in his study of lipids found in nuclei (inclusive of nuclear membranes); also by Dounce (54) for his study of the contained enzymes.

If unkilled tissue is washed in saline and minced in an high-speed mixing machine, then centrifugated, and the processes repeated on the centrifugal precipitate, masses of microscopic fibers are collected that give every indication of being the chromosomes from the nuclei. This is the material used by Mirsky and Pollister (134) in some of their chemical studies.

Zittle and Zitin (224, 225) liberated bull sperm heads by supersonic vibrations, a method which Chambers and Flosdorf (35a) devised and Sevag and Smolens (176) also used for the mechanical disintegration of bacteria.

Laskowski (98) isolated erythrocyte nuclei with the aid of lyolecithin made by treating lecithin with an extract of bee-sting glands.

Modern chemical analyses confirm the early conclusion that chromatin is a compound of nucleic acid with a protein. In animals, the predominant nucleic acid of chromatin seems to be universally desoxyribonucleic acid (49, 61, 134), more specifically thymonucleic acid in the form of a very high polymer (177). Most animal nuclei have little or no nucleic acid of other sorts. The same acid may be the one that predominates in most vegetable nuclei, for Feulgen's test, specific for the presence of desoxyribose in the nucleic acid, is positive in most plant nuclei, as it is for animal chromatin (22). Even the nuclear granule in yeast gives this test, although there are, here and there, various

plants whose nuclei give a negative response. Each mononucleotide of thymonucleic acid is a very flat structure (5), measuring 15×7.5 Å. In the natural polymeric condition they are stacked to occupy 3.34 Å each, so that a megamolecule having 2000 units, such as is reported from many animal nuclei, would be about 6700 Å (*i. e.*, 0.67 μ) long.

Chromatin of fish spermatozoa is considered to be thymonucleic acid in combination with a very nearly equal number of equivalents of protamine. The latter is a basic protein of low molecular weight or, more precisely, a filamentous polypeptide chain containing, in various species of fish, from 15 to 33 amino acids each. The basicity of protamine is due almost entirely to its arginine content; as this amino acid comprises typically two out of every three of the residues present, the protamine nucleate contains as a balanced salt only a little over half as much protamine as nucleic acid by weight.

Nuclei from immature fish testes have some protamine together with histone (134). Histone is a class of protein only less basic than protamines, being rich in the three characteristic basic amino acids. Their molecule size is reported as corresponding to a minimum Svedberg unit, *viz.*, a molecular weight of about 16,000 to 17,000 and their count of amino acid residues presumably 144. We have no sure reports of whether their molecule is compact or filamentous. It is supposed, however, that the protein of hemoglobin is also histone; and x-ray diffraction studies indicate that hemoglobin has nonfilamentous molecules (147).

Salmon erythrocyte nuclei, as well as liver nuclei, contain histone and no protamine, within the limits of chemical detection.

Nuclei from veal thymus gland (123) have at least three proteins: a protein of the histone type; a sulfur-rich protein with a slightly acidic isoelectric point; and a protein of globulin type, not particularly sulfur-rich. No protamine has been detected in thymus gland, or in any mammalian tissue thus far. It is uncertain how many of the proteins of thymus nuclei occur in combination with the thymonucleic acid, and how many are proteins present without prosthetic groups, except that undoubtedly all the histone is present as nucleoprotein.

It is hardly yet certain what kind of bond exists between the nucleic acid and its associated protein. As early as the pioneer studies of Miescher (130) and Burian (30a), it was argued that there are two contrasted kinds of union, which, following Burian, one could designate as protein nucleate and as protein–nucleic acid, respectively. Protein-nucleic acid was understood to be a firm, nondissociable combination of the nucleic acid with protein (in the case of salmon sperm, more specifically with a part of the protamine), possessing some highly acidic phosphoric atoms still unreplaced. If hydrolyzed, it would reunite in a loose salt, *viz.*, a protein–hydrogen–nucleate. Chromatin, according to Burian, was a protamine salt of protamine-nucleic acid, capable of losing its first protamine on the slightest acidulation, but requiring hydrolysis to lose the rest of its protamine. The majority of modern investigators do not corroborate Burian, but Sevag and Smolens (176) have offered evidence that at least some nucleoproteins from bacterial sources show other than salt bondings between the nucleic acid and the protein.

Nucleic acid is not continuously present in all stages of all germ cells (33). This provides an argument against considering it an essential part of the differential pattern of a gene, though it does not diminish the probability that it is an essential item in the machinery for some of the things that a gene must do. It is also incidental evidence for a loose bonding, possibly merely as a salt between it and the essential proteins of chromatin. Moreover, the persistent structure of the chromosome depends on its protein,

not on its nucleic acid.   This inference is further corroborated by experiments by Mazia and Jaegar (121, 122), who digested away all nucleic acid from a giant salivary chromosome of *Drosophila* without disorganizing the chromosome.   The converse experiment, of keeping the nucleic acid and digesting away the protein (Caspersson, 33) causes the chromosome to disintegrate.

Very recently Stedman and Stedman (188a) announced that a class of proteins to which they give the name "chromosomin" is the predominant constituent of the chromosomes of all vertebrates.   Apparently on account of a high content of dicarboxylic amino acids these proteins have an isoelectric point lying between pH 3 and 5, even though they contain some 25% of basic amino acids.   Their solubility is low.   With hematoxylin and other nuclear stains they show the familiar colors of stained nuclei.   It is not yet clear whether or not either or both of the acidic proteins reported by Mayer and Gulick (123) are identifiable as examples of chromosomin.

Glycogen is a known constituent of the nucleus, being found at times in recognizable granules (90, 132).   Although no hexose has been identified as belonging to chromatin, it seems self-evident that carbohydrate must play a large part in the synthetic processes that go on in the chromosomes, and that the nuclear glycogen must serve as the carbohydrate in these processes.   As we can hardly expect the completed glycogen to traverse the nuclear membrane, we suppose that a mechanism for polymerizing it out of glucose-1-phosphate must exist within the nucleus, very possibly within the chromosomes.

As the functions of genes must be essentially enzymatic, some light upon the problem of genes may be expected from the facts discovered about enzymes.   Various pure crystallized enzymes are now known, and certain of them have been studied by the Svedberg method, as well as by x-ray diffraction, e. g., by Bernal and Crowfoot (16).   All of them seem to be nonfibrous proteins, except for the special case of myosin, a fibrous muscle protein having certain enzyme characters.   This presents us with a likelihood, but by no means a certainty, that gene protein agrees with virus protein in being nonfibrous.   Most enzymes seem to work by virtue of an enzymatic activating surface which makes a multiple-point contact with the substrate (14).   Our thought respecting the active face of the gene conforms to the multiple-contact idea.   In fact it presupposes a monolayer surface of protein consisting of nothing but multiple-contact points, the total assemblage of which gives an adequate résumé of all that is contained in the gene protein.

Enzymes and related catalytic substances are often closely associated with certain divalent and trivalent metallic ions, or they may include these metals firmly bound into organic compounds.   A microincineration of nearly any biological preparation leaves a pattern of white ash, major

portions of which correspond to chromatin in general form and location (8, 152a, 171, 195a). The metal principally represented is calcium. Samples of chromatin, as obtained by the Behrens method, are found to contain calcium and magnesium in the following percentages by fat-free dry weight (Williamson and Gulick, 201a):

	Calcium	Magnesium
Veal thymus chromatin, %..................	1.3–1.4	0.07–0.1
Human tonsil chromatin (1 multiple sample), %.............................	1.25	0.07
Chromatin from bovine supermammary lymph node (1 sample), %...........	1.41	0.06

Both of these minerals are three or four times as concentrated in the total nucleus as in the cytoplasm of the above examples of lymphoid tissue.

Iron, as is clearly demonstrated by the iron-hematoxylin histological stain, has an extraordinary *post mortem* affinity for chromatin. This fact impairs or invalidates most of the early attempts to learn the distribution of iron within the live cell.

Yakusizi, using his methods for separating the nuclei out of avian blood cells (220), attempted to determine the nuclear iron content (218, 219). As his figures indicate a cytoplasmic iron content up to 140 times as concentrated as that of the nuclei, it is evident that a distressingly small contamination with cytoplasm could affect the nuclear figures very heavily; hence, that erythrocytes are a disadvantageous starting material for this test. Yakusizi then proceeded to a study of human pus. He states that, in these cells, the nuclear iron concentration is substantially less than the cytoplasmic. The figures in his tables, however, yield for nuclei a dry-weight concentration varying from 0.7 to 4.2 mg. per 100 g., and for cytoplasm, from 0.56 to 5.4 mg. per 100 g. Such a spread in the figures raises the suspicion that he is one more analyst who has struggled in vain against the extraordinary difficulties of keeping out all extraneous iron. If such is the case, his minimum figures are the ones most likely to represent the true conditions, and the nuclei may be credited with as little as 0.7 mg. per 100 g. dry weight—or perhaps even some of this may also represent contamination.

Zittle and Zitin (224, 225) broke up bull spermatozoa by supersonic vibrations, and sorted the heads, midpieces, and tails by centrifugation. They recognize that their procedure was not entirely safe against artifactitious iron, but believe that their best batches, on which they report, were essentially uncontaminated. Whatever unrecognized trace contaminations may occur will inevitably have their chief effect in the sperm head fraction. These authors find, in terms of dry weight:

	Average wt. per 100 g., mg.
Total spermatozoa..........................	7.1
Sperm heads................................	4
Sperm midpieces...........................	20
Sperm tails................................	10

About 60% of the total figure was in the nonhematin fraction, leaving 40% as hematin, presumably cytochromes. The spectrum of cytochrome c could not be found, however.

Enzyme study led them to the belief that iron-carrying oxidative enzymes (cytochromes) are present in every part, but in only minimal concentration in the heads. The scarcity of nuclear iron is in line with the observation by Bunding (29) that catalase, a cytochrome (iron-containing) enzyme, is found in the cytoplasm but not in the nucleus or chromosomes of *Chironomus* salivary gland cells.

The only other metal that we find definitely reported present in nuclei is zinc (218, 219). In human pus nuclei, Yakusizi found about 5 mg. to 9 mg. per 100 g. dry weight. The concentration is nearly always several times as high as in the cytoplasm, as Yakusizi states clearly, and as will be seen as soon as some arithmetical slips occurring in his table have been set right.

As for alkali metals, no evidence for sodium has been reported from any nuclear preparation, and some nuclei seem certainly to have none (*cf.* Gulick, 79). Potassium (115) also has not been definitely recognized, but there is some circumstantial evidence for its probable occurrence (30, 79).

We find, then, that disregarding sodium and potassium, for which reliable procedures are unknown, approximately 94 to 96% of the metallic content of chromatin is calcium, most of the rest is magnesium, and the only other metals reported are iron and zinc, in mere traces. All four of these are elements known to have significant roles in catalytic complexes. Nuclear calcium must add up to be no small percentage of the body's total non-skeletal calcium. Similarly a very substantial fraction of the body's minute but indispensable quota of zinc would seem to be located in the nuclei. One might also comment that a region in which a scarce, indispensable substance is concentrated, is most probably a location where it is indispensable; but nothing is known definitely about the value of any of these elements in the nucleus.

The enzymes Dounce (54) found in the nuclei of rat-liver cells are given in the accompanying tabulation:

Enzyme	Remark
Alkali phosphatase	More concentrated than in total liver
Arginase	
Cytochrome oxidase	
Esterase	Less abundant than in total liver
Lactic acid dehydrogenase	
Acid phosphatase	
Catalase	Almost lacking
Succinic acid dehydrogenase	
Cytochrome c	Low or absent
Coenzyme I	

Riboflavin or its compound, the "yellow enzyme," seemed also to be low or absent. As compared with the cytoplasm of the same cells, the enzyme yield of the nuclei thus far seems to run low.

An outstanding characteristic of the chromonema, or string of genes present in the chromosome, is its predilection for a doubled condition,

while, on the other hand, under most conditions it seems to resist aggregating on a larger scale. The string of genes grows metabolically into two duplicate strings, then stops, and until the two have separated seems incapable of doubling again. In the synaptic stage of the ripening of the germ cells we have another example of the doubled condition, although arrived at by conjugation instead of by fission. Possibly we have here another example of the same enigma presented by protein megamolecules, which refuse to grow to an indefinite size like crystals, but after repeating identical parts a certain exact number of times are under the necessity of closing off their molecule.

Several authors have argued about the forces involved in such behavior. Friedrich-Freksa (64) reported the strength of the magnetic field by which two identical chromonemata might be expected to attract each other, on the supposition that all polar elements come into parallel alignment in a direction at right angles to the long axis of the chromonena. He concluded that an adequate force was available to draw together the pairing chromosomes, provided the medium was at the isoelectric point of the chromatin, so as to avoid all electrostatic repulsion. As soon as an electrostatic charge is established, all mutually similar structures will repel each other. Delbrück (50) argues for a similar conception, but adds the thought that the semiquinone condition which his theory of protein synthesis calls for, at the moment when the two-step oxidative synthesis is half completed, is a source of extra electrons, hence of an electrostatic charge; and he suggests that this may be the moment at which daughter chromosomes in a cell division begin to repel each other and move off to the loci of the daughter nuclei. Both of these authors look on the attractions and repulsions as phases of the same type of electrochemical happenings, closely associated with the problem of the synthesis of a duplicate set of genes. In this they seem to differ from Fabergé (56), who looks for a somewhat longer range force to draw the homologous chromosomes together for their synapse, and suggests what is known as the "Guyot-Bjerknes" effect, which can establish an attraction between synchronously pulsating bodies.

VI. Genes as Chemical Mechanism

Earlier investigators never thought of doubting that any gene must comprise an organized complex of many molecules. More recently, it has been proposed that each gene is a single molecule (51, 52, 136–153). Lastly, it has been contended .that any entire chromosome is so deeply bound that it is but one molecule, and that the genes are blocks within this supergiant molecule (cf. 67–70, 95, 96). It is a little doubtful, however, just what these several opinions may mean when applied to such aggregates of nucleoprotein as the chromosomes.

A crystal is an aggregate of indefinite size, composed of atoms bonded together into one complex. Qualitatively, it is the same when very small as when grown to large size. "Molecule" means nothing in the description

of a crystal of sodium chloride, although in describing a solution it has a very definite meaning. As applied to units within a crystal of a complex carbon compound it has more meaning, but it is not always used according to the same definition. Bonds of some sort but of very dissimilar grades tie together every atom participating in the formation of any crystal. There are several possible approaches to the use of the term "molecule" to a constituent within a crystal:

1. if it is an aggregate that exists as a separate molecule when the substance of the crystal is brought into solution;

2. if there is reason to suppose that any further subdivision would cause qualitative alteration of the "molecule";

3. Svedberg's studies leading to the recognition of chemical particles of definite size, now known as megamolecules, and which may hold their identity even within a crystal;

4. viruses leading also to the recognition, at least in theory, of a definite sized, definitely ordered atom complex comprising a minimum functional unit of autosynthetic substance, probably identifiable with the Svedberg megamolecule of this substance.

As there is no question on hand of bringing genes into solution, criterion 1 has no direct applicability. Apparently the other three criteria influence different authors in different relative degrees. Criterion 2, when applied to living material, is open to reservations; an intact dog is qualitatively different from a decapitated dog, but it would be a bold biochemist who would attempt to define the difference in terms of the rupturing of a megamolecule. Criterion 3 has an explicit meaning in solutions, but in undissolved aggregates it amounts to an analogy and an inference, ideologically valuable, probably, but somewhat difficult to render precise. Criterion 4 seems to embody a useful concept, despite the fact that self-reduplication of genes is subject to limitations not found in viruses, so that a gene is much less unconditionally capable of autonomous reproduction than is a megamolecule of virus. Despite all this, it still seems to be a serviceable hypothesis to suppose that each gene has some of the characteristics implied in calling it one molecule—a unit of definite size and atomic content, with some degree of unitary chemical autonomy, and unable to change its content or arrangement of atoms or its state of aggregation without becoming a different "molecule," a different substance, and more or less a different gene. Such an hypothetical identification of a gene with a megamolecule, if significant at all, as I truly suppose (and many geneticists with me) should take on more manifold meanings as our knowledge of both genes and megamolecules progresses.

Any gene possesses just two explicit functions: self-reduplication according to a particular, very precise set of rules; and production of non-genic, biologically active material, presumably an enzyme protein characteristic of the particular gene. Within the framework of these activities must be found the entire means of control exercised by genes not only over hereditary transmission from one generation to the next, but also for the steps in the embryology of organ formation and tissue specialization. Obviously, the sum total of the genes (the genom), a double set of which is found in each cell of every tissue, must be highly versatile in what it can do; yet we do not know whether individual genes are versatile or not. One gland tissue produces one enzyme, another gland produces some other. Is this because in the different tissues different genes are permitted to "do their trick," or is it that differing cytoplasms induce the same gene to yield a slightly different product? One is tempted to suppose that each gene produces just one active principle (193), and that the differing outcome in the diverse tissues of the body is because this first active product induces more or less different results when it works on different cytoplasmic substrates. This *a priori* supposition may, or may not, be true. It is conceivable even if not too plausible, that feeding a different substrate to the gene may induce it to alter the nature of its own primary product. But considering how much less in most respects the nuclear environment of the genes varies than the cytoplasmic setup, we may well assume tentatively that no one gene has very great versatility in its primary products.

The gene's primary products are unknown to us. For the most part, the multitudinous enzymes we find abundantly present in tissues and organs are doubtless not the ones that were produced by genes. The list of enzymes found thus far in the nucleus is not impressive, and except for the nuclear phosphatase they are in rather scant concentration. Seemingly what the genes produce are formative enzymes, doubtless largely of the sort involved in the synthesis of protein molecules, not much known to science, representing early links in the chains of causation that lead up to the later synthesis in the cytoplasm of our more familiar cytoplasmic enzymes. In any case, we know that the ontogenetic outcome of gene action is a highly complex set of tissues and organs, and that the explanation must have to do with changes in the over-all metabolic effects of genes when functioning in biologically different environments.

In rehearsing the items to be found in the environment that must influence the metabolic behavior of genes we note:

1. Other genes and their products. When we consider how reliably all the genes and chromosomes of a cell keep time with each other in their

cycle of cell division, it is evident that their timing is not accidental or independent. One is impelled to consider the possibility, for example, of pace-setting genes which influence the pace of the rest. Certain parts of the gene machinery—the "heterochromatin" portion—seem to have a particularly strong influence upon the supply of nucleic acid; and in consequence an oversupply of the heterochromatin segments in the chromosomes can accelerate the mitotic cycle. It is also reported that a gene not normally located in contact with heterochromatin will have an altered influence upon the heredity if it is translocated into a heterochromatin region.

2. Interactions between cytoplasm and genes. In the formation of specialized tissues there is no alteration, as far as we can tell, in the store of genes, but merely a specialized interaction between the gene supply and a particular variety of cytoplasm. (This statement deliberately ignores certain rare exceptional cases of loss of parts of chromosomes, as in the worm, *Ascaris megalocephala*, because such events seem to be unrepresentative.) Characteristically, a tissue corresponds to a steady, self-perpetuating state set up between the gene and the cytoplasm, the latter so constituted that it induces the genes to perpetuate the same type of cytoplasm and cellular aggregate. The most obvious exemplification of this may be found in tissue cultures, tissue grafts, and malignant metastases.

3. Reaction between gene and neighboring or remote tissue products ("hormones"). Whenever a hormone alters tissue metabolism and growth, as, for example, when estrogen causes proliferation of uterine epithelium, when ovarian hormones in poultry suppress cock feathering, or when the embryo retina stimulates formation of the embryo lens, our interpretation must be that certain active circulating compounds, produced in a particular tissue by virtue of the gene–cytoplasm steady state in that location, intrude upon the gene–cytoplasm steady state of a different tissue and cause it to be reconstituted on an altered plan. In many such effects, the primary alteration is in the timing of some sort of timed reaction. Some particular synthesis or specialized oxidation is speeded or retarded, so that at a crucial moment in the history of the tissue or of the individual the adequate or, up to that moment, inadequate formation of a crucial substance will tip the balance toward one or the other of the contrasted end results. Extensive genetic evidence for this type of effect has been collected particularly by Goldschmidt (67–70).

The statement seems valid, then, that all development, specialization, tissue differentiation, and temporal changes observable in the ontogeny of a multicellular organism are accomplished through cytoplasmic specializa-

tions, which are responsible for differentiated steady states of the gene-cytoplasm complex. Ultimately, however, these states are subject to evolutionary control through genes, and through natural selection of genes, because the cytoplasm is itself subject to gene regulation.

VII. Chemical Phenotypes

Investigations by Beadle and Tatum (11, 193), on a species of the ascomycete, *Neurospora*, start with the proposition that each gene presumably has one chemical enzymatic function, and that it will be enlightening to obtain samples of gene functions. They have discovered three defect mutations of a metabolic nature: one a strain that differs from the normal *Neurospora* by its inability to synthesize pyridoxine (vitamin B_6); another that cannot synthesize the thiazole ring portion of thiamine (vitamin B_1); and a third strain distinguished by inability to supply itself with p-aminobenzoic acid. Each strain must have its particular vitamin supplement added to its nutrient medium. With that on hand, its growth and its morphology are indistinguishable from those of the normal ("wild") strains.

The last of these strains is particularly interesting. It has no difficulty in the synthesis of tyrosine or phenylalanine. Tyrosine and hydroxybenzoic acid will not offset a lack of p-aminobenzoic acid, nor will m- or o-aminobenzoic acid. p-Nitrobenzoic acid will suffice, and, less efficiently, even aniline. The enzyme omitted from its physiology must be concerned with amination in the para position.

Pigments comprise a favorable material upon which to test out the manner of action of genetic "unit characters." Prominent among the pigments which give color to plant blossoms are the flavonol and related anthocyanin (or flavylium salt) pigments, having the general structure:
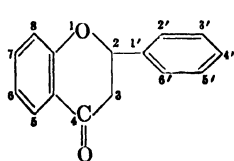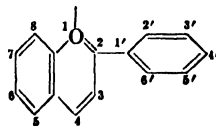
Flavone   Flavylium (an anthocyanin)

On this molecular framework, positions 3, 5, 7, and 4' are regularly hydroxylated; positions 3' and 5' can also take an hydroxyl group; and hydroxyl groups at positions 7, 3', and 5' are likely to be altered into methoxy groups. Furthermore hydroxyl groups on positions 3 and 7 are open to forming glucosidic unions with certain carbohydrates; *cf.* papers by

Lawrence and by Scott-Moncrieff, jointly (101, 102) and separately (172–174). A large variety of genetically controlled colors in the range of the oranges, reds, and purples are thus made possible. The genetics of these colors has been studied in several genera among which *Streptocarpus* may be cited as representative. *Streptocarpus* has three known pairs of alleles dealing with flavylium colors, as follows:

R   provides position 3′ with hydroxyl;
r   fails to do so;
O provides position 5′ with hydroxyl;
o   fails to do so;
D   places one hexose each on positions 3 and 5; and
d   leaves hydroxyl 5 uncombined, but places an hexose–pentose disaccharide complex on position 3.

As each allele pair deals with a different part of the flavylium rings, it has an independent action on the molecule, with the following results:

R O D is blue;
R o D is magenta;
R O d is mauve;
R o d is rose;
r o D is pink; and
r o d is salmon.

The Rr and the Oo pairs are understood to be indivisible single genes. It is possible that Dd represents a closely associated gene complex.

The color varieties of maize provide a most favorable material for mutation studies (157, 162, 178–180); at the same time the plant is not unfavorable for cytologic study (124). As compared with animals, the flowering plants have an advantage for students of gene mutations through the fact that, in passing from generation F_1 to F_2, a few nuclear generations are gone through in a haploid condition, thus sifting out the vast majority of chromosome deficiencies, very few of which are haplo-viable. Maize has the additional merit that, from the kernels of a single ear, one may obtain excellent statistics on the seed characters produced by any particular cross. Colors involving the aleurone, the pericarp, and the stalk, leaves, and roots belong largely to the flavonol and anthocyanin series, for which a variety of regulatory genes has been found. Some of these determine the chemistry of the pigment, and others its location in the plant or its relative intensity in different locations. The dominants R, C, and A_2 and the recessive i locate color in the aleurone. The dominant P causes

it to show in the pericarp. Dominants B and Pl cause it to show in the main parts of the plant. Dominant A gives the pigment the anthocyanin structure, chrysanthemin, while its recessive allele, when homozygous, leads to the flavonol homologue, isoquercitrin. As both the A series and the R series include many alleles, the genetic possibilities are multitudinous. Just a few of them are shown in Table III. In the R series of alleles is the gene R^r which behaves on further mutation as if it had two mutable centers whose mutations are independent of each other, or else as if two genes existed which were independent of each other except for being mechanically inseparable. Much study of these color genes is in progress, some of it facilitated by the discovery of a gene "Dt" (157) which accelerates the mutation rate of some, but not all, of the alleles in the A series.

TABLE III

GENETIC POSSIBILITIES IN MAIZE

Allele	Aleurone color, R, C, A₂ or i being present	Pericarp color, P being present	Plant color, B or Pl being present
A^b	Purple	Brown (dominant)	Purple
A	Purple	Red	Purple
a^p	Pale	Brown (dominant)	Red-brown
a	Colorless	Brown (recessive)	Brown

Butenandt (31), Kikkawa (89), and other workers (see 151), including Beadle, Anderson, and Maxwell (10), have been interested in the colors of insect eyes and chitinous structures. The work of Beadle and associates homologized some of the eye color effects in *Drosophila*, in *Habrobracon*, and *Ephestia*—the latter a parasite living on *Habrobracon*. They found that "vermillion" and "cinnabar" in *Drosophila* eye color are defects which are overcome by feeding the Drosophila with extracts of "wild" *Habrobracon*. The interpretation is that the missing enzymes cease to be needed for production of the "wild" color in *Drosophila* if the finished products of the enzyme action are in the food. Extracts from "red " or "cantaloupe" *Habrobracon* are also effective. "Orange" or "ivory" *Habrobracon* are effective in correcting "vermillion" *Drosophila*, but have no effect on cinnabar. "White" is essentially the same. Extension of this type of experiment leads to the conclusion that "cinnabar" of *Drosophila*, "orange" of *Habrobracon* and probably the Aa alleli of *Ephestia* are biochemical homologues.

Kikkawa (89) made comparable studies of *Ephestia*, *Bombyx*, and *Drosophila*, with the results shown in Table IV. As the table indicates, he

<div align="center">TABLE IV</div>

<div align="center">PIGMENT IN EGGS AND VARIOUS ORGANS</div>

Source	Enzyme			
	I	II	III	IV
Ephestia				
Wild (black)	+	+	+	+
Red	−	±?	+	+
Bombyx				
Black (wild)	+	+	+	+
White I	+	+	−	+
Pink	+	+	+	−
Brown 2-K	±	±	±	±
Drosophila				
eyes:				
Wild	+	+	+	+
Vermillion	−	±?	+	+
Cinnabar	+	+	−	+
White	±	+	±	−
	tryptophane	hydroxytryp-tophane	kynurenine	chromogen
	↓	↓	↓	↓
	α-oxytryp-tophane	kynurenine	chromogen	chrome

interprets the results in terms of the presence or absence of the genes for four enzymes presiding over four reactions:

Tryptophane ⟶ hydroxytryptophane ⟶ kynurenine ⟶

"+ chromogen," presumably related to kynurenic acid ⟶ "+ chrome."
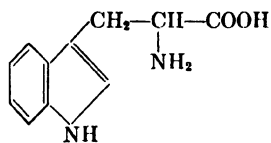
Tryptophane

Hydroxytryptophane

Kynurenine

Kynurenic acid

As tabulated by Kikkawa, the effects of some genes deal sharply with the presence or absence of single enzymes (*Bombyx,* white I; *Bombyx,* pink; *Drosophila,* cinnabar) while other genes seem to have an effect that is spread over more than one mode of action (*Bombyx,* brown 2-K; *Drosophila,* white).

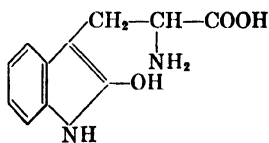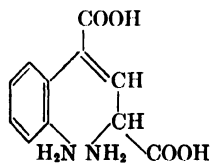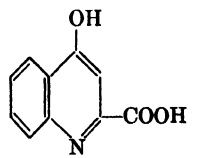Work with insects gives the impression that some genes with miscellaneous effects exist, as exemplified by the two last-mentioned alleles. Mammalian studies carry this impression much farther. Castle (35) has analyzed the correlations between guinea pig body size and the presence or absence of various well-known hair color genes, and concludes that there is a degree of correlation not explainable by chance. In other words, the hair color genes may be described as "polyurgic" genes affecting among other things color in the manner of Mendelian alternatives, and affecting body size additively, along with numerous other genes.

The melanin class of pigments, which are chiefly responsible for the darker colors in mammalian hair, are derived chemically from phenylalanine and from tyrosine (155) by way of their oxidation product, dihydroxyphenylalanine ("dopa") and derivatives involving further

Tyrosine

"Dopa"

5,6-Dihydroxy-
indole-2-carboxylic acid

5,6-Dihydroxyindole
(melanin precursor)
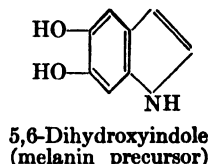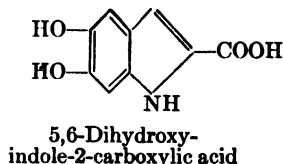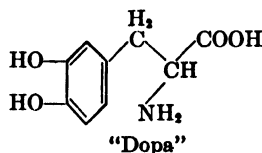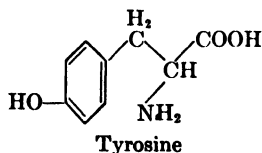
oxidation with the formation of additional ring complexes. Mammals with the genetic consitution for heavily pigmented skin and hair have an enzyme in the lower epidermis that converts dopa into black pigment almost instantly. This reaction is influenced in guinea pigs by several pairs of alleles (161). The Aa pair, in which A is responsible for the zoning of different color shades on the tip, midportion, and base of each hair (in the "wild" manner) are genes without influence on the dopa reaction. Spotted animals (homozygous "ss") give a powerful dopa test in the pigmented area, but no reaction in skin from a white spot. The recessive of the C gene (cc) gives an animal which lacks this dopa reaction

entirely in either skin or hair follicles. The recessive of the P gene (pp) gives full pigmented hair and white skin. The skin has no dopa test, except in the hair follicles. Alleles of the F gene produce various degrees of dilution of hair color, and influence the dopa enyzme potency in the follicles. Thus, an ff animal exhibits scarcely any dopa reaction, and has almost albino hair. It appears that, in the case of the skins of mammals, one pigmentation enzyme, the dopa oxidase, has its concentration and its distribution determined by a whole series of genes. At the same time, as Castle (35) has pointed out, these genes are also influencing other characters such as the animal's size. Obviously the chemical consideration of these genes has answered a few questions and raised a multitude of new questions.

No species is better explored for biochemical phenotypes than is man. But relatively few of the discovered chemical anomalies have been sufficiently clarified to cast light on the subject of chemical genetics. Only two examples will be touched upon here.

Cystinuria, or the persistent overexcretion of the amino acid cystine, is a congenita[1] defect which generally behaves as a Mendelian recessive. It appears to be a defect mutation involving the proper metabolism of various amino acids. Feeding of cystine itself or of homocystine, which has, like cystine, the S—S group, does not lead to increased cystine elimination; but the —SH amino acids, cysteine and homocysteine (125), as well as the C—S—C amino acid, methionine, yield excretory cystine in the cystinuric patient. Excreted cystine is largely endogenous, and various amino acids will stimulate its production. The output induced by taking alanine almost equals that from eating cysteine (81). Glycine and glutamic acid have about half as much effect as alanine. Proteins with a biologically good amino acid content seem to facilitate the normal use of sulfur amino acids by the cystinuric (109). There seems to be considerable difference between different individual patients, and the responses vary at different levels of protein metabolism.

Hemophilia, like color blindness, is considered a classic example of an X-chromosome recessive, inherited by boys exclusively from (usually heterozygotic) mothers, and theoretically seen phenotypically in girls only if the father is hemophilic and the mother carries the recessive trait. This condition is virtually never fulfilled, as hemophilic boys seldom live to marriageable age; so it is usually stated that women never show hereditary hemophilia. The defect resides in the blood plasma (113, 114), and concerns neither prothrombin, antithrombin, nor fibrinogen. It can be corrected passively for a few hours by injection of the euglobulin fraction out of normal human blood. The material passes through a Berkefeld filter.

VIII. Experiments on the Relation of Nucleus and Cytoplasm

Several investigators have shown much ingenuity in their attacks on the problem of the relation between nucleus and cytoplasm. Baltzer (7) fertilized the eggs of various species of newts with the sperm of other species,

then removed the female pronucleus, leaving the cytoplasm of one species with a haploid nucleus of another. The result was in each case temporarily viable, but the embryos perished either earlier or later in consequence of abnormalities that were fairly characteristic of the respective combinations. He concluded that such interspecies experiments bring out an astonishing number of maladjustments between the materials from the two species, thus revealing the great number of aspects in which harmony is requisite.

Working by a similar technique, Porter (153) studied the influence of nucleus and cytoplasm on the temperature adjustments of physiological races of the frog, *Rana pipiens*. The different races, when in their natural habitats, develop at comparable speeds despite the large differences in the temperatures that surround them. But if a northern race was hatched under southern temperature conditions, it was overspeeded, or the southern race raised in the north was overretarded. The eggs, if deprived of the female pronucleus, could develop without difficulty into haploid tadpoles. But if the pronucleus represented a more northern variety than that of the cytoplasm, it instigated a badly regulated acceleration in which the forward parts of the body became more accelerated and proportionately abnormally large. If, on the other hand, the nucleus was from the more southern race and the cytoplasm northern, there was retarded cell division and the forward parts were undersized in comparison with the hinder parts. Pennsylvanian nuclei, being of intermediate latitude, behaved as northern in relation to Florida and as southern in relation to Vermont, thus giving opposite morphological disparities with the same set of genes because of their changed relations to the cytoplasm. Such facts as these make it evident that gene action can only be formulated adequately in terms of the gene–cytoplasm complex.

IX.　Genes and Biological Evolution

From the standpoint of evolutionary theory one must suppose a primitive early stage of life in which the organism consisted of one gene (or several repeats of one gene) and its perquisites. We see approximately this state of affairs in the case of viruses, although we must guard against thinking of viruses, with their obligate parasitism, as truly primitive forms of life.

Progress beyond this stage inevitably soon involved a plurality of genes, and the whole history of many-celled organisms must undoubtedly have called for frequent increases in the gene count as the organism advanced

toward greater complexity.   In sexually reproducing plants and animals, a well-known source of extra chromosomes or chromosome segments is found in the various possible accidents and irregularities in the processes of reduction, maturation, and fertilization.   Botanists are familiar with polyploid varieties and species among many genera of flowering plants (48, 55).   These happenings are evidently quite an adequate source of more chromosomes and more genes for plants.   In animals, the process is by no means so obvious, as polyploid races of animals are rare in nature or in the laboratory.   Fankhauser (58, 59) made a study of newt larvae to obtain a gage of the frequency of chromosome irregularities.   He found that between 1 and 2% of all the larvae examined showed extra chromosomes or groups of chromosomes.   In addition to amphibia, he cites the observation of polyploid individual animals among mosquitoes, fruit flies, silk worms, etc., so we may state confidently that new supplies of chromosomes and of their contained genes are as available among animals as among plants, whenever the exigencies of evolution call for them, and without any need for their creation *de novo*.   The difference between plants and animals on this score seems to be that, in plants, the fixation of races carrying the polyploid mutations is facilitated through their ability for self-pollenation and vegetative reproduction (see Huxley, 84).

It has been pointed out that the constant process of gene mutation must eventually transform supernumerary sets of genes into more elaborate sets of nonduplicate genes.   As purely supernumerary genes are of doubtful value to the organism, there is little to protect such genes from being lost through new accidents (84, pp. 455, 476).   So a double process must gradually sort out the genes brought in through polyploidy—some are transformed into useful and eventually necessary new genes, while many are lost entirely.   Probably in many cases a nonessential gene is one which has come in as a duplicate and has not yet evolved into an indispensable item.

The concept of the gene as a chemical entity containing a vast but finite number of atoms carries with it the proposition that the number of mutations which any one gene can undergo is inconceivably vast, yet fundamentally finite.   While the extreme number of possible mutations is so great, those that occur easily will comprise not more than a moderate list; hence the statistical likelihood that any mutation observed as occurring once will be observed again if only a sufficient watch is kept (see 51, 52; and 83, 140, 152).   The frequency of the occurrence will be determined purely by the chemical facility with which it can occur.   But the frequency with which a particular living form is found represented in nature is entirely a different matter, since that depends also on the ability of the

mutant stock to perpetuate and multiply. Thus natural populations are the resultant of two unrelated factors—mutation rate, conforming to the chemical rules governing the chemistry of gene substance, and survival value, having to do with the adjustments of the organism and its environment. The two factors pull statistically in different directions, and the final outcome is seen as an adaptive compromise constantly approaching the optimum that can be achieved under the conditions set by the chemical rules. Any long-standing population which has undergone rigorous natural selection may be expected, in consequence of the natural selection, to possess a set of genes about as favorable as the chemical make-up of its genetic pattern will easily permit (see, e. g., Huxley, 84). This makes it inevitable that very nearly any new mutation will be other than beneficial simply because any random change from a condition that is already a practical optimum is all but fated to be a useless or detrimental change. Furthermore, the vast majority of chemical alterations of an enzymatic molecule are in the direction of impaired potency, and only a trivial minority are toward higher potencies or newly developing potencies. We have consequently two possibilities which occur with far greater frequency than all others:

1. Mutation in a useful gene usually means a detrimental alteration; and the stock carrying the mutant will tend to die out through selective elimination.

2 Mutation in a gene that the organism does not need usually lowers the gene's potency; and after enough such mutations have occurred the gene has virtually become cancelled out of the hereditary system through the accumulation of degenerative changes.

The greatest part of what geneticists observe comes within these two categories, and has therefore no relationship to the progressive evolution of the species. Only when a species faces adjustment to an unfamiliar environment are conditions favorable for the occurrence of fairly numerous adaptively advantageous mutations.

The fact that every cell carries a full quota of every kind of gene means that any gene may conceivably affect the character of any organ in the body. A geneticist labels a gene as responsible, let us say, for a particular eye color, an effect which it produces by the formation of a particular oxidative enzyme. The same gene will probably produce the same enzyme in other parts of the body also, leading to an oxidative effect that has some very different visible effect. Although fundamentally such a gene may be doing the same thing in both places, to the experimental observer the gene will have a multiple effect; for convenience we may well

designate such a gene as "polyurgic," *viz.*, responsible for various effects in various anatomical regions (144). Polyurgic genes introduce a particularly interesting phase of genetic evolution. If most genes are polyurgic, then traits of a fairly general type, like body size, leg length, etc., will be more or less influenced one way or another by almost any gene that one may care to bring under consideration. This can be tested experimentally.

As we have already mentioned, Castle (35) compared the sizes of mammals bearing various color genes, and found that a high proportion of these genes have definite influence on the body size in mice, rats, and rabbits. Schwab (170) studied a number of genes that affect the external characters of *Drosophila*, testing their effect, if any, on the spermatheca of the female. He found that, in seven of the mutations, there were effects upon the shape of the spermatheca which worked in an additive manner. One mutation showed a nonadditive interaction effect. Another mutation was intermediate between the additive and nonadditive classes. Two of the genes studied were without detectable effect on the spermatheca. Both investigators concluded that genes tend to have multiple effects.

Where one gene apparently produces in different parts of the body effects which do not seem to stand in any rational relationship to each other, several alternative explanations are possible. First, it is not always a simple matter to demonstrate that the gene being experimented upon is really indivisible, and not a cluster of closely associated genetic units. Again, it is conceivable that processes which look to us today quite unrelated may, on further analysis, prove to be dependent on the same biochemical prerequisite. A pigment effect and a hormone effect may both depend on the power to complete a particular aromatic ring, and it may be long in the future before we can hope to demonstrate the identity of the gene action in the two seemingly irrelevant effects. And, finally, it may eventually be discovered that a single gene molecule under two contrasted conditions may actually catalyze two different processes which, even to the fully informed chemist, will still stand as dissimilar catalyses. This last alternative should be the last one for a scientist to accept, as it would constitute the least simple explanation; but in the sharply diversified tissues of mammals, or even of fruit flies, there is much more risk that this explanation may finally have to be invoked than can be the case with the scantly differentiated tissues of an ascomycete. Hence comes one of the advantages of experimenting with the ascomycetes (see Beadle and Tatum, 11). "Polyurgic" genes, then, are so named only for present convenience. As science progresses, many of them should be reinterpreted on the basis of some underlying single biochemical effect.

Natural selection must accept or reject a gene on the basis of its entire

effect on the power of the organism to perpetuate itself. Theoretically, a gene may be responsible at once for certain advantageous and certain disadvantageous traits. If the advantages are great enough, the gene may get favorable selection despite serious disadvantages. *Homo sapiens* differs from nearly all other mammals in his hereditary lack of uric acid oxidase in the liver, which in other mammals converts the uric acid into the nonacidic, much more water-soluble and harmless allantoin. This deficiency renders us liable to become victims of gout, a disease impossible for the other mammals to have. Does the uric oxidase gene somehow stand in the way of evolutionary progress in other mammals through some not yet discovered polyurgic feature or was it just a casual coincidence that a weakness toward gout was saddled upon a biological stock that was otherwise too superior to perish?

As a corollary to multiple-functioning genes, we inevitably have the problem of characters influenced by many genes, as illustrated by the converse aspect of Castle's studies of body size (35). Probably all but the very simplest characters are influenced by various genes, and may consequently be referred to as "polygenic" traits (47, 118, 119). Fisher (63) has shown that polygenic traits, varying as they do over a relatively smooth frequency curve, undergo modification through natural selection according to a special set of mathematical rules, but that the practical outcome of selection of polygene characters differs but little from the effect of natural selection on genetically simple Mendelian characters.

Polygenic characters are often thought of as addition effects and doubtless some of the time that is correct. Theoretically two genes, or two enzymes from two genes, may either produce two chemically unrelated effects that add their results together, or may influence two aspects of the same fundamental chemical process, in which case very probably they will affect the end results not additively but in proportion to the product of their separate effects multiplied together. Genes influencing the body size of mammals are often treated as additive. Doubtless many of them stand in that relation to each other; and in any case, where the percentage alteration is small, the experiment will not distinguish between a sum and a product. If two genes each cause a 5% increase, do the two together give a 10% increase, or is the true relation the product, 1.05×1.05, which equals 1.1025? No biological experiment can distinguish between the two possibilities. But where the proportional change is far greater, the distinction may make an experimental difference. If each gene alteration doubles the size of an organ, will the two genes acting simultaneously additively treble the organ, or by multiplication make it quadruple? Using

tomato fruit as a test case, it was found (116) that the aggregate effect of the genes was in proportion to the products of their separate effects. This means that although the genes were not alleles to each other, they were altering mutually interdependent aspects of one major biochemical process in such a way that the reaction induced by each gene enhanced the effectiveness of the other.

Biological evolution, we are often reminded, is an irreversible process. Despite the marine origin of the vertebrates, for example, it is impossible for a mammal to become a fish. If it does revert toward its previous condition, what it becomes is a seal or a whale, never a fish. Quite otherwise, however, with a gene, as it may very credibly reverse the last mutation it has suffered, perhaps in some cases even the last two or three mutations. On the basis of chemical plausibility we may expect to find some mutations reversible and others definitely irreversible. For example, a mutation that robs the gene of a piece of its synthetic pattern would doubtless be quite impossible to turn back to the condition that had the pattern. But if the mutation consists of a shift in the location of some relatively small group or even in the insertion or deletion of a fairly simple chemical group, it is to be expected that, whenever the necessary activating energy for the reaction is locally present, the reverse mutation may easily occur. Various genes have been successfully followed through a mutation followed by a reverse mutation (52). With many more genes, nothing of this sort has been observable. Such differences will doubtless continue to be recorded.

X. Chemical and Paleontological Concepts

It is conceivable that a particular chemical alteration of one part of a gene may start a train of influences favoring comparable alterations in other of its parts, and perhaps even parallel changes in neighbor genes. If the effect of these changes is beneficial, or harmless, or not too harmful, we may see over a period of time a sort of predestined evolution which tends to run its course even without the guidance of selection, or perhaps running the stock finally to extinction through uncontrolled exaggeration of traits which have become disadvantageous. This, if it is a correct analysis of fact, might underlie the "orthogenesis" for which so many paleontologists believe they find historical evidence. All other types of evidence are rather conspicuously absent thus far (see Huxley, 84), but eventually it should become possible for geneticists to put orthogenesis to an experimental test.

Various paleontologists have long been of the opinion that phyla can

doom themselves to extinction through overspecialization. There are several aspects to such a thesis. The meaning might be that specialization carries a risk, *viz.*, that the organism has in effect placed a wager on future events, and takes the risk of betting on the wrong thing, on an environment that is destined to disappear too quickly to permit a new adjustment. But a successful bet is also in the possibilities. Gregory and Raven (74) point out that, if specialization *per se* is fatal, then the particular group of paleozoic fishes that became the ancestors of the terrestrial quadruped forms were even before that event excellent candidates for extinction. Their bet, however, proved to be on a winning combination. Another possibility under the designation "specialized" may be a variant of orthogenesis, it being assumed that orthogenetic tendencies actually occur in evolutionary history. If an orthogenetic tendency piles up into establishing a vicious circle, the result might easily be nonadaptive "specialized" modification to the point of race extinction. Surely, if the orthogenetic principle exists, that must be one of the ways in which it might behave. Still again, in terms of genetics, "specialization" could be interpreted as the possession of a peculiar set of genes. If altered circumstances brought a need for eliminating or inhibiting these genes, the question arises whether the organism is capable of doing without them—whether their elimination would not constitute a lethal mutation. Viewed from this slant, "overspecialization" could mean the possession of indispensable genes which are potentially detrimental. The evolutionary cure, in cases that are curable, would be successive step-down mutations, followed by progressive adjustment in the rest of the gene system. Genetically speaking, then, overspecialization does not look like a major danger in the evolutionary process.

It may be commented, in conclusion, that the direct and explicit aid rendered by chemistry to the understanding of genetics and biological evolution has been rather limited, though with the promise of more to come; but that aid by way of supplying patterns of thought and pertinent analogies has already become very important.

Bibliography

1. Alexander, A. E., *Proc. Roy. Soc. London*, **A179**, 470 (1941).
2. Anson, M. L., and Stanley, W. M., *J. Gen. Physiol.*, **24**, 769 (1941).
3. Astbury, W. T., *Proc. 7th Intern. Genetic. Congr.*, **1939**, 49.
4. Astbury, W. T., *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, p. 63.
5. Astbury, W. T., and Bell, F. O., *Nature*, **141**, 747 (1938).

6. Astbury, W. T., and Bell, F. O., *Nature,* **147,** 696 (1941).
7. Baltzer, F., *Naturwissenschaften,* **29,** 177, 196 (1940).
8. Barigozzi, C., *Zellforsch. mikroskop. Anat.,* **26,** 462 (1937).
9. Barnard, J. E., *J. Roy. Microscop. Soc.,* **59,** 1 (1939).
10. Beadle, G. W., Anderson, R. L., and Maxwell, J., *Proc. Natl. Acad. Sci. U. S.,* **24,** 80 (1938).
11. Beadle, G. W., and Tatum, E. L., *ibid.,* **27,** 499 (1941).
12. Beale, G. H., *J. Genetics,* **42,** 197 (1941).
13. Behrens, Martin, *Z. physiol. Chem.,* **209,** 59 (1932).
14. Bergmann, Max, *Harvey Lectures.* Vol. 31, Williams & Wilkins, Baltimore, 1936, p. 37.
15. Bergmann, Max, and Niemann, C., *Science,* **86,** 187 (1937).
16. Bernal, J. D., and Crowfoot, D., *Nature,* **133,** 794 (1934).
17. Bernal, J. D., and Fankuchen, I., *J. Gen. Physiol.,* **25,** 111 (1941).
18. Bernal, J. D., Fankuchen, I., and Riley, D. P., *ibid.,* **142,** 1075 (1938).
19. Bertrand, G., *Bull. Soc. Chem. Biol.,* **18,** 213 (1936).
20. Bertrand, G., and Macheboeuf, M., *Compt. rend.,* **182,** 1305 (1926).
21. Bertrand, G., and Vladesco, R., *ibid.,* **173,** 176 (1921).
22. Boas, F., and Biechele, O., *Biochem. Z.,* **254,** 467 (1932).
23. Bolomey, R. A., and Allen, F. W., *J. Biol. Chem.,* **144,** 113 (1942).
24. Bourdillon, J., *J. Gen. Physiol.,* **25,** 263 (1941).
25. Brand, E., Cahill, G. F., and Block, R. J., *J. Biol. Chem.,* **110,** 399 (1935).
26. Brand, E., Cahill, G. F., and Kassell, B., *ibid.,* **125,** 415 (1938).
27. Brinkhous, K. M., *Am. J. Med. Sci.,* **198,** 509 (1939).
28. Buchmann, W., and Timoféeff-Ressovsky, N. W., *Z. Indukt. Abstamm. Vererbungslehre,* **71,** 335 (1936).
29. Bunding, I. M., *J. Cellular Comp. Physiol.,* **17,** 133 (1941).
30. Bureau, V., *Arch. intern. physiol.,* **39,** 311 (1934).
30a. Burian, R., *Ergeb. Physiol.,* **5,** 768 (1906).
31. Butenandt, A., Weidel, W., and Becker, E., *Naturwissenschaften,* **28,** 63 (1940).
32. Calvin, M., Kodani, M., and Goldschmidt, R., *Proc. Natl. Acad. Sci. U. S.,* **26,** 340 (1940).
33. Caspersson, T., *Skan. Arch. Physiol.,* **73,** Suppl. No. 8 (1936).
34. Caspersson, T., *Chromosoma,* **1,** 147 (1939).
35. Castle, W. E., *Genetics,* **26,** 177 (1941).
35a. Chambers, L. A., and Flosdorf, E. W., *Proc. Soc. Exptl. Biol. Med.,* **34,** 631 (1937).
36. Chambers, L. A., and Henle, W., *ibid.* **38,** 481 (1941).
37. Chambers, L. A., and Henle, W., *J. Exptl. Med.,* **77,** 265 (1943).
38. Churney, L., *Cytology, Genetics, and Evolution.* Univ. Pennsylvania Press, Philadelphia, 1941, p. 113.
39. Claude, A., *Trans. N. Y. Acad. Sci.,* **4,** 79 (1941).
40. Cohen, S. S., and Stanley, W. M., *J. Biol. Chem.,* **142,** 863 (1942).
41. Cohen, S. S., and Stanley, W. M., *ibid.,* **144,** 589 (1942).
42. Cole, P. A., and Sutton, E., *Cold Spring Harbor Symposia,* **9,** 66 (1941).
43. Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.,* **124,** 543 (1938).
44. Crossmon, G., *Science,* **85,** 250 (1937).
45. Crowfoot, D., *Nature,* **135,** 591 (1935).

46. Darlington, C. D., *Nature*, **149**, 66 (1942).
47. Darlington, C. D., *ibid.*, **150**, 154 (1942).
48. Darlington, C. D., *ibid.*, **150**, 404, 574 (1942).
49. Davidson, J. N., and Waymouth, C., *ibid.*, **152**, 47 (1943).
50. Delbrück, M., *Cold Spring Harbor Symposia*, **9**, 122 (1941).
51. Demerec, M., *J. Heredity*, **24**, 369 (1933).
52. Demerec, M., *Cold Spring Harbor Symposia*, **9**, 145 (1941).
53. Dolley, D. H., *J. Med. Research*, **21**, 95 (1909).
54. Dounce, A. L., *J. Biol. Chem.*, **147**, 685 (1943).
55. Fabergé, A. C., *J. Genetics*, **33**, 365 (1936).
56. Fabergé, A. C., *ibid.*, **33**, 383 (1936).
57. Fabergé, A. C., *ibid.*, **43**, 121 (1942).
58. Fankhauser, G., *J. Morphol.*, **68**, 161 (1941).
59. Fankhauser, G., *Proc. Natl. Acad. Sci. U. S.*, **27**, 507 (1941).
60. Fankuchen, I., *J. Gen. Physiol.*, **24**, 315 (1941).
61. Feulgen, R., *Z. physiol. Chem.*, **165**, 215 (1927).
62. Fischer, F. G., and Hultsch, K., *Biochem. Z.*, **299**, 104 (1938).
63. Fisher, R. A., *Nature*, **150**, 54 (1942).
64. Friedrich-Freksa, H., *Naturwissenschaften*, **28**, 376 (1940).
65. Gates, R. R., *J. Roy. Microscop. Soc.*, **58**, 97 (1938).
66. Glaser, O., *Trans. N. Y. Acad. Sci.*, **4**, 5 (1941).
67. Goldschmidt, R., *Physiologische Theorie der Vererbung.* Springer, Berlin, 1937.
68. Goldschmidt, R., *Quart. Rev. Biol.*, **3**, 307 (1928).
69. Goldschmidt, R., *The Material Basis of Evolution.* Yale Univ. Press, New Haven, 1940.
70. Goldschmidt, R., and Kodani, M., *Am. Naturalist*, **76**, 529 (1942).
71. Goodrich, H. B., Hill, G. A., and Arrick, M. S., *Genetics*, **26**, 573 (1941).
72. Gowen, J. W., *Cold Spring Harbor Symposia*, **9**, 187 (1941).
73. Green, A. A., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **142**, 447 (1942).
74. Gregory, W. K., and Raven, H. C., *Ann. N. Y. Acad. Sci.*, **42**, 273 (1941).
75. Gulick, A., *Proc. Mo. Acad. Sci.*, **3**, 75 (1937).
76. Gulick, A., *Quart. Rev. Biol.*, **13**, 140 (1938).
77. Gulick, A., *Proc. 7th Intern. Genetic. Congr.*, **1939**, 135.
78. Gulick, A., *Growth*, **3**, 241 (1939).
79. Gulick, A., *Botan. Rev.*, **7**, 433 (1941).
80. Hahn, L., and Hevesy, G., *Nature*, **145**, 549 (1940).
81. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **142**, 3 (1942).
82. Huggins, M. L., *J. Chem. Phys.*, **8**, 598 (1940).
83. Hutt, F. B., and Mueller, C. D., *Am. Naturalist*, **77**, 181 (1943).
84. Huxley, J., *Evolution: The Modern Synthesis.* Harper, New York, 1942.
85. Janssen, L. W., *Protoplasma*, **33**, 410 (1939).
86. Kalckar, H. M., *Chem. Revs.*, **28**, 71 (1941).
87. Kaplan, R., *Naturwissenschaften*, **28**, 79 (1940).
88. Kausche, G. A., *ibid.*, **27**, 212 (1939).
89. Kikkawa, H., *Genetics*, **26**, 587 (1941).
90. Klestadt, W., *Frankfurt. Z. Path.*, **4**, 444 (1910).
91. Knapp, E., Reuss, A., Risse, O., and Schreiber, H., *Naturwissenschaften*, **27**, 304 (1939).

92. Knight, C. A., *J. Biol. Chem.*, **145**, 11 (1942).
93. Knight, C. A., and Laufer, M. A., *ibid.*, **144**, 411 (1942).
94. Knight, C. A., and Stanley, W. M., *ibid.*, **141**, 39 (1941).
95. Koltzoff, N. K., *Biol. Zentr.*, **48**, 345 (1928).
96. Koltzoff, N. K., *Biol. Zhur.*, **7**, 3 (1938). Eng. resumé, pp. 44–46.
97. Kossel, A., *The Protamines and Histones*. Longmans, Green, London and New York, 1928.
98. Laskowski, M., *Proc. Soc. Exptl. Biol. Med.*, **39**, 354 (1942).
99. Laufer, M. A., *J. Biol. Chem.*, **143**, 99 (1942).
100. Lawrence, W. J. C., Price, J. R., Robinson, G. M., and Robinson, R., *Trans. Roy. Soc. London*, **B230**, 149 (1939).
101. Lawrence, W. J. C., and Scott-Moncrieff, R., *J. Genetics*, **30**, 155 (1935).
102. Lawrence, W. J. C., Scott-Moncrieff, R., and Sturgess, V. C., *ibid.*, **38**, 299 (1939).
103. Lazarow, A., *J. Biol. Chem.*, **138**, lxxv (1941).
104. Lea, D. E., and Catcheside, D. D., *J. Genetics*, **44**, 216 (1943).
105. Leipert, Th., and Kurokawa, T., *Biochem. Z.*, **261**, 425 (1933).
106. Levene, P. A., *J. Biol. Chem.*, **126**, 63 (1938).
107. Levene, P. A., and Bass, L. W., *Nucleic Acids*. Chemical Catalog Co., New York, 1931.
108. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **109**, 623 (1935).
109. Lewis, H. B., Brown, B. H., and White, F. R., *ibid.*, **114**, 171 (1936).
110. Linderstrøm-Lang, K., *Ann. Rev. Biochem.*, **8**, 37 (1939).
111. Loring, H. S., *J. Biol. Chem.*, **123**, lxvi (1938).
112. Loring, H. S., *J. Gen. Physiol.*, **25**, 497 (1942).
113. Lozner, Eugene L., Kark, R., and Taylor, F. H. L., *J. Clin. Investigation*, **18**, 603 (1939).
114. Lozner, Eugene L., and Taylor, F. H. L., *ibid.*, **18**, 821 (1939).
115. Macallum, A. B., *J. Physiol.*, **32**, 95 (1905).
116. MacArthur, J. W., *J. Heredity*, **32**, 291 (1941).
117. Mather, K., *Proc. Roy. Soc. London*, **B124**, 97 (1937).
118. Mather, K., *Nature*, **149**, 731 (1942).
119. Mather, K., *Biol. Rev. Cambridge Phil. Soc.*, **18**, 32 (1943).
120. Mazia, D., *Cold Spring Harbor Symposia*, **9**, 40 (1941).
121. Mazia, D., and Jaeger, L., *Proc. 7th Intern. Genetic. Congr.*, **1939**, 212.
122. Mazia, D., and Jaeger, L., *Proc. Natl. Acad. Sci. U. S.*, **25**, 456 (1939).
123. Mayer, D. T., and Gulick, A., *J. Biol. Chem.*, **146**, 433 (1942).
124. McClintock, B., *Genetics*, **23**, 315 (1938).
125. McKinney, H. H., *J. Heredity*, **28**, 51 (1937).
126. McKinney, H. H., *Proc. 7th Intern. Genetic. Congr.*, **1939**, 200.
127. Mensikai, S. W., *J. Roy. Microscop. Soc.*, **59**, 82 (1939).
128. Metz, C. W., *J. Heredity*, **26**, 177 (1935).
129. Metz, C. W., *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 23 (1941).
130. Miescher, F., *Die histochemischen und physiologischen Arbeiten*. Vol. 2, Vogel, Leipzig, 1897.
131. Miller, G. L., and Andersson, K. J. I., *J. Biol. Chem.*, **144**, 459 (1942).
132. Mironesco, Th., *Compt. rend. soc. biol.*, **67**, 731 (1909).

133. Mirsky, A. E., *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, p. 1.
134. Mirsky, A. E., and Pollister, A. W., *Proc. Natl. Acad. Sci. U. S.*, **28**, 344 (1942).
135. Miyake, N., *Keijo J. Med.*, **4**, 247 (1933).
136. Muller, H. J., *Am. Naturalist*, **56**, 32 (1922).
137. Muller, H. J., *Proc. 1st Intern. Congr. Plant Sci.*, **1929**, 897.
138. Muller, H. J., *Proc. Natl. Acad. Sci. U. S.*, **21**, 16 (1935).
139. Muller, H. J., *Am. Naturalist*, **69**, 405 (1935).
140. Muller, H. J., *Cold Spring Harbor Symposia*, **9**, 151 (1941).
141. Muller, H. J., and Altenberg, E., *Proc. Soc. Exptl. Biol. Med.*. **17**, 10 (1919).
142. Muller, H. J., and Painter, T. S., *Z. Indukt. Abstamm. Vererb.*, **62**, 316 (1932).
143. Muller, H. J., and Prokofyeva, H. A., *Compt. rend. acad. sci. U. S. S. R.*, **4**, 74 (1934).
144. Neel, J. V., *Am. Naturalist*, **76**, 630 (1942).
145. Neurath, H., *J. Phys. Chem.*, **44**, 296 (1940).
146. Newcombe, H. B., *J. Genetics*, **43**, 145 (1942).
147. Perutz, M. F., *Nature*, **150**, 324 (1942).
148. Petrie, A. H. K., *Biol. Rev. Cambridge Phil. Soc.*, **18**, 105 (1943).
149. Pfeiffer, H. H., *Nature*, **143**, 335 (1939).
150. Philpot, J. St. L., and Eriksson, J. B., *ibid.*, **132**, 932 (1933).
151. Plagge, E., and Becker, E., *Biol. Zentr.*, **58**, 231 (1938).
152. Plough, H. H., *Cold Spring Harbor Symposia*, **9**, 127 (1941).
152a. Policard, A., *Bull. histol. appl. physiol. path. tech. microscop.*, **17**, 238–245 (1940).
153. Porter, K. R., *Trans. N. Y. Acad. Sci.*, **4**, 213 (1942).
154. Raffel, D., and Muller, H. J., *Genetics*, **25**, 541 (1940).
155. Raper, H. S., *Fermentforschung*, **9**, 206 (1927).
156. Reiss, P., *Compt. rend.*, **179**, 641 (1924).
157. Rhoades, M. M., *Cold Spring Harbor Symposia*, **9**, 138 (1941).
158. Rittenberg, D., *ibid.*, **9**, 283 (1941).
159. Ross, A. F., *J. Biol. Chem.*, **143**, 685 (1942).
160. Ruska, H., *Naturwissenschaften*, **28**, 45 (1940).
161. Russell, W. L., *Genetics*, **24**, 645 (1939).
162. Sando, C., Milner, R. T., and Sherman, M. S., *J. Biol. Chem.*, **109**, 203 (1935).
163. Sax, K., *J. Gen. Physiol.*, **25**, 533 (1942).
164. Schmidt, W. J., *Arch. Exptl. Zellforsch. Gewebezücht.*, **19**, 352 (1937).
165. Schmidt, W. J., *Chromosoma*, **1**, 252 (1939).
166. Schmitt, F. O., *Physiol. Revs.*, **19**, 270 (1939).
167. Schultz, J., *Proc. 7th Intern. Genetic. Congr.*, **1939**, 257.
168. Schultz, J., *Cold Spring Harbor Symposia*, **9**, 55 (1941).
169. Schultz, J., *Ann. Rev. Physiol.*, **5**, 35 (1943).
170. Schwab, J. J., *Genetics*, **25**, 157 (1940).
171. Scott, G. H., *Proc. Soc. Exptl. Biol. Med.*, **38**, 30 (1940).
172. Scott-Moncrieff, R., *J. Genetics*, **32**, 117 (1936).
173. Scott-Moncrieff, R., *Perspectives in Biochemistry*. Cambridge Univ. Press, London, 1937, p. 230.
174. Scott-Moncrieff, R., *Ergeb. Enzymforsch.*, **8**, 277 (1939).
175. Sealock, R. R., and Silberstein, H. E., *Science*, **90**, 517 (1939).
176. Sevag, M. G., and Smolens, J., *J. Biol. Chem.*, **140**, 833 (1941).

177.   Signer, R., Caspersson, T., and Hammersten, E., *Nature*, **141**, 122 (1938).
178.   Stadler, L. J., *Proc. 6th Internat. Genetic. Congr.*, **1**, 274 (1932).
179.   Stadler, L. J., *Proc. 7th Internat. Genetic. Congr.*, **1939**, 269.
180.   Stadler, L. J., *Cold Spring Harbor Symposia*, **9**, 168 (1941).
181.   Stanley, W. M., *Science*, **81**, 644 (1935).
182.   Stanley, W. M., *Phytopathology*, **25**, 899 (1935).
183.   Stanley, W. M., Report at meetings of A.A.A.S., Princeton, Dec. 1936.   See *Science News Letter*, Jan. 9, 1937, p. 20.
184.   Stanley, W. M., *Proc. Am. Phil. Soc.*, **77**, 447 (1937).
185.   Stanley, W. M., *Medicine*, **18**, 431 (1939).
186.   Stanley, W. M., *J. Gen. Physiol.*, **25**, 881 (1942).
187.   Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, **139**, 325 (1941).
188.   Stanley, W. M., and Knight, C. A., *Cold Spring Harbor Symposia*, **9**, 255 (1941).
188a.   Stedman, E., and Stedman, Ellen, *Nature*, **152**, 267 (1943).
189.   Stoneburg, C. A., *J. Biol. Chem.*, **129**, 89 (1939).
190.   Straub, J., *Naturwissenschaften*, **31**, 97 (1943).
191.   Svedberg, T., *Nature*, **128**, 999 (1931).
192.   Swanson, C. P., *J. Gen. Physiol.*, **26**, 485 (1943).
193.   Tatum, E. L., and Beadle, G. W., *Proc. Natl. Acad. Sci. U. S.*, **28**, 234 (1942).
194.   Thoday, J. M., *J. Genetics*, **43**, 189 (1942).
195.   Troland, L. T., *Am. Naturalist*, **51**, 321 (1917).
195a.   Uber, F. M., *Botan. Rev.*, **6**, 204–225 (1940).
196.   du Vigneaud, V., *Cold Spring Harbor Symposia*, **9**, 275 (1938).
197.   Vinson, C. G., Mo. Agr. Expt. Sta., *Research Bull.* **237** (1936).
198.   Vlès, F., and Novel, S., *Arch. phys. biol.*, **1**, 301 (1922).
199.   Vlès, F., Reiss, P., and Vellinger, E., *Compt. rend.*, **179**, 349 (1924).
200.   Wang, Cheng-Fa, and Wu, Hsien, *Chinese J. Physiol.*, **15**, 67 (1940).
201.   Warburg, O., *Z. physiol. Chem.*, **70**, 413 (1910).
201a.   Williamson, M. B., and Gulick, A., *J. Cellular Comp. Physiol.*, **20**, 116 (1942).
202.   Wipf, L., and Shackelford, R. M., *Proc. Natl. Acad. Sci. U. S.*, **28**, 265 (1942).
203.   Wrinch, D. M., *Nature*, **134**, 978 (1934).
204.   Wrinch, D. M., *Protoplasma*, **25**, 550 (1936).
205.   Wrinch, D. M., *Nature*, **138**, 651 (1936).
206.   Wrinch, D. M., *Proc. Roy. Soc. London*, **A160**, 59 (1937).
207.   Wrinch, D. M., *Science*, **85**, 566 (1937).
208.   Wrinch, D. M., *Nature*, **139**, 972 (1937).
209.   Wrinch, D. M., *Phil. Mag.*, **25**, 705 (1938).
210.   Wrinch, D. M., *Proc. 7th Intern. Genetic. Congr.*, **1939**, 329.
211.   Wrinch, D. M., *J. Genetics*, **40**, 359 (1940).
212.   Wrinch, D. M., *Phil. Mag.*, **31**, 177 (1941).
213.   Wrinch, D. M., *Nature*, **150**, 270 (1942).
214.   Wrinch, D. M., and Jordan-Lloyd, D., *ibid.*, **138**, 758 (1936).
215.   Wu, H., *Chinese J. Physiol.*, **5**, 321 (1931).
216.   Wu, H., and Wang, C. F., *J. Biol. Chem.*, **123**, 439 (1938).
217.   Wynd, F. Lyle, *J. Gen. Physiol.*, **25**, 649 (1942).
218.   Yakusizi, N., *Keijo J. Med.*, **7**, 276 (1936).
219.   Yakusizi, N., *ibid.*, **7**, 289 (1936).
220.   Yakusizi, N., *ibid.*, **7**, 521 (1936).

221. Yasui, K., *Cytologia*, **9,** 120 (1938).
222. Zamenhof, S., and Demerec, M., *Am. Naturalist,* **77,** 380 (1943).
223. Zittle, C. A., and O'Dell, R. A., *J. Biol. Chem.,* **140,** 899 (1941).
224. Zittle, C. A., and Zitin, B., *ibid.,* **144,** 99 (1942).
225. Zittle, C. A., and Zitin, B., *ibid.,* **144,** 105 (1942).

.

SPECIFICITY, CLASSIFICATION, AND MECHANISM OF ACTION OF THE GLYCOSIDASES

By

WILLIAM WARD PIGMAN

Washington, D. C.

CONTENTS

I. Introduction

The decade 1920–1930 was a period of intense activity in the investigation of the action of the glycosidic hydrolyzing enzymes, but the main emphasis was laid on studies of the kinetics of the reaction. The following decade was marked by a shift in emphasis to studies of the specific action of these enzymes and of the difference between enzymes hydrolyzing the same substrates but derived from various sources. The glycosides furnish very excellent substrates for such investigations because they are fairly easily prepared, are susceptible to many variations in structure, and are

41

usually well-defined crystalline compounds. The investigations of Helferich and associates, in particular, on the action of the β-glucosidase of almond emulsin on aryl and alkyl β-glucosides provide one of the most widespread studies made of the influence of structure on the rate of a chemical reaction.

Unfortunately, it is difficult to compare the results of different workers in the field, as each worker usually selects his own experimental conditions which frequently are not directly comparable with those of other investigators. For a discussion of this problem, see reference 53. In the work which will be presented, most of the comparisons are made at a substrate concentration of 0.052 M, and the ease of hydrolysis is indicated as the enzyme efficiency (*Wertigkeit*), which is calculated from the experimentally determined reaction constant by use of the relation

$$\text{Enzyme efficiency} = \textit{Wertigkeit} = k/(g \times \log 2)$$

where k is the first order reaction constant at 30° C., calculated with minutes as the time unit, and g represents the number of grams of emulsin in 50 ml. of reaction mixture.

II. Mechanism of Action

It is generally postulated that enzymatic action takes place through the intermediate formation between enzyme and substrate of a complex which decomposes into enzyme and hydrolytic products. This concept was amplified by von Euler, who suggested that, for the formation of the enzyme-substrate complex, two areas on the enzyme are required (5). A postulated mechanism based on these assumptions is illustrated in Figure 1 for the hydrolysis of an alkyl glucoside (52).

In the figure, the two oval areas represent active areas on the surface of the enzyme on which the glycoside is adsorbed, the aglycon group* being adsorbed on one area and the sugar radical on the other. It is assumed that area I exhibits extremely specific adsorption and area II, general adsorption. The term "adsorption" is used in a very general sense; and the combination may take place through hydrogen and electrostatic bonds, van der Waals' forces, and possibly even weak covalent bonds. As has been shown by Hitchcock and by Weidenhagen, the same kinetic equations result from a consideration of the formation of the complex as a dissociable chemical compound or as a simple adsorption (41, 46, 66).

According to the proposed mechanism, the first stage of the reaction takes place with the adsorption of the substrate on the two areas of the

---

* Glycosides may be represented as A—O—Gl. The radical A is the aglycon group; the corresponding alcohol or phenol is the aglycon. Gl represents the glycosyl or sugar radical.

enzyme. The enzyme–substrate complex then adds a molecule of water (or an hydronium ion) at the glucosidic linkage. Disruption of the glucosidic linkage probably next takes place with the formation of a complex consisting of the enzyme, the sugar, and the aglycon. The final stage comprises the dissociation of the products of hydrolysis from the surface of the enzyme.

According to the mechanism postulated, enzymatic hydrolysis differs from acid hydrolysis only in the formation of an enzyme–substrate complex; and the actual cleavage of the glucosidic linkage is brought about by hydronium ions (or solvent molecules) in both types of hydrolysis. The
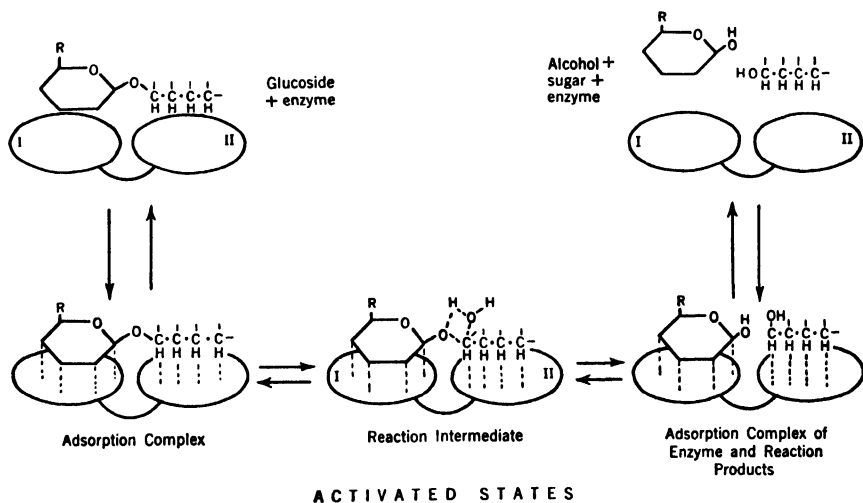
Fig. 1.—Mechanism for enzymatic hydrolysis of an alkyl glucoside.

role of the enzyme is probably to furnish a preliminary activation of the substrate molecule. This preliminary activation would account for the well-known observation that the activation energies of enzyme-catalyzed reactions are much smaller than those of the corresponding acid-catalyzed reactions (see Table V, page 56). The total activation energy might be considered to be derived from the initial activation which takes place in the formation of the enzyme–substrate complex and from the subsequent addition of the solvent molecules or of hydronium ions. During the period of combination of enzyme and substrate, which probably is very short (46), the translational and vibrational motions of the substrate molecule are restricted and the corresponding energies may provide one

source of the activation which takes place in the first stage of the reaction. The decrease in entropy which takes place in this step provides evidence for this conception (61). For this type of activation, the energy would be derived primarily from the kinetic energy of the substrate molecule. Activation might also result from molecular distortion of the substrate molecule by the enzyme (3, 8, 48). Thus, in the formation of the enzyme–substrate complex, the glucosidic linkage might be "strained," or this "straining" might take place by movement of the two active areas of the enzyme relative one to the other. A portion of the activation energy may be provided in this way at the expense of the energy of the enzyme molecule.

It is difficult, except in simple cases, to interpret the effects of substitution and structural changes on enzymatic hydrolysis because of a lack of knowledge of the several variables involved. It would seem that two factors are particularly important: the strength of the glycosidic bond undergoing hydrolysis; and the strength and position of the bonds between the substrate and enzyme. The first might be considered to parallel the ease of acid hydrolysis or it might be evaluated by comparing the ease of hydrolysis at equal concentrations of the enzyme–substrate complex.

Some investigators claim that a direct comparison of rates of enzymatic hydrolysis under arbitrary conditions, as made in the following pages, is not justified, and claim that the comparisons can only be made at equal concentrations of the enzyme–substrate complex (cf. Veibel and Lillelund, 64). The writer takes the point of view that both types of comparisons are valuable and should be made where possible. It must remain for the future to decide which is the more valuable method and whether the great amount of additional work required in determining the dissociation constants is necessary. It should be noted that, when the ease of hydrolysis at equal concentrations of the enzyme–substrate complex is compared, the most important factor of enzyme-catalyzed reactions, i. e., the ease of formation and stability of the complex, probably is eliminated from consideration.

The second factor cannot be readily evaluated, although the Michaelis dissociation constant should give some information. According to the mechanism presented, it would be expected that the most easily hydrolyzed substrates are those with the weakest glycosidic linkages and with a balanced adsorption of the substrate and desorption of the products of hydrolysis. When the bonds between the substrate and enzyme are too strong, the dissociation of the products of hydrolysis would seem to be the limiting factor. It would also be anticipated that the bonds between the substrate and enzyme, located closest to the glycosidic oxygen bridge, would be more effective in promoting the hydrolysis than those more remote from the glycosidic linkage undergoing hydrolysis.

From the standpoint of the mechanism of the reaction, the comparison of the ease of enzymatic hydrolysis of the phenyl and thiophenyl β-glucosides is of particular interest. These compounds differ only in the nature of the glucosidic linkage which is, in one case, through an oxygen atom and, in the other, through a sulfur atom. Presumably, the adsorptive forces between the enzyme and substrate are very similar, since the aglycon groups and the sugar radicals of the two are identical. A study (51) of the action of almond emulsin on these substances shows that, although phenyl β-glycoside is easily hydrolyzed (E.E. = 33,000 $\times$ 10^{-5}), thiophenyl β-glucoside is not appreciably affected (E.E. < 10^{-5}). It seems probable that this great difference should be ascribed to differences in the addition of water or hydrogen ions to the glycosidic linkage of the enzyme–substrate complex, since there is a similar marked difference in the ease of acid hydrolysis of the two compounds.

As an explanation of the specific action of the enzymes, this mechanism will be applied in a qualitative fashion; but it is hoped that time may be found in the future to apply it as a quantitative explanation of the mechanism of inhibition and of pH effects.

III. Enzymes of Almond Emulsin

1. Preparation and Purification

Since almond emulsin is a mixture of enzymes and other material, the enzymes present in any particular preparation will depend on the preparatory procedure followed, and possibly on the source of the almond nuts. Various methods are described for this purpose (2, 45, 62). A typical procedure involves crushing and defatting the nuts, extracting the enzymes with water, and precipitating the aqueous extract with alcohol to give the powder known as almond emulsin. Most of the specificity studies have been carried out with the *Rohferment* of Helferich (39).

The *Rohferment* may be further purified, particularly by the application of adsorption procedures, until the activity increases some 15 times (β-glucosidase value 15 to 16). In spite of intensive efforts, greater activity has not been achieved; but this purified product still is probably a mixture of at least several enzymes. Simple treatment of solutions of the *Rohferment* with activated carbon (30), in limited quantities, results in the preferential removal of certain enzymes (α-galactosidase) and other materials and the activity is increased about threefold (β-glucosidase value about 3). If this purified solution is then treated with tannin, a product is obtained with about 10 times the original activity (β-glucosidase value about 10). The same result is obtained by adsorption of the enzymes of *Rohferment* on silver hydroxide. This "silver-purified" emulsin is easily prepared; and considerable work has been carried out with it, particularly

studies of the constitution of the enzymes present. Additional purification can be obtained by adsorption on and elution from silica gel (30) or, more conveniently, by treatment of solutions of the "silver purified" emulsin with activated carbon. The most active material has a β-glucosidase value of 16 (17, 30).

2. Enzyme Constituents

In addition to β-glucosidase, which hydrolyzes β-glucosides, other glycosidases are present in almond emulsin. In general, the principal method utilized for proving the presence of other enzymes is based on the assumption that, if enzymes other than β-glucosidase are present, purification procedures should change the relative proportions of the various enzymes. If the relative ease of hydrolysis of two glycosides by the *Rohferment* is quite different from that of a purified preparation, it is probable that two different enzymes are involved. Thus, activated carbon partially removes from *Rohferment* solutions the component responsible for the hydrolysis of α-galactosides; tannin purification, mentioned above, increases the ability of almond emulsin to hydrolyze α-mannosides as compared with β-glucosides (55). The presence of an α-galactosidase and an α-mannosidase activity different from the β-glucosidase activity was postulated prior to the work cited, since similar differences can be produced by the selective destruction of one of the components. When almond emulsin solutions are heated, or exposed to ultraviolet lignt, the ability to hydrolyze α-mannosides is retained much better than that for hydrolyzing β-glucosides (11, 21, 22).

The foregoing evidence is interpreted as proving the existence of an α-galactosidase and an α-mannosidase, in addition to the β-glucosidase of almond emulsin. By similar methods, the probable existence of two other glycosidases has been demonstrated (22, 36). These are β-glucuronisidase hydrolyzing β-glucuronides and β-(N-acetyl)-glucosaminidase hydrolyzing the β-glycosides of N-acetylglucosamine.

Almond emulsin also hydrolyzes β-D-xylosides, L-arabinosides and α-D-lyxosides. The available evidence indicates that the enzymes responsible are β-glucosidase, α- and β-galactosidase, and α-mannosidase, respectively. This might be expected because the pentosides differ from the corresponding hexosides only in the substitution of a hydrogen for the terminal primary alcoholic group of the hexosides (see diagrams on page 51). As shown in Table I, purification of the *Rohferment* increases the ability of the emulsin to hydrolyze α-L-arabinosides and β-xylosides in approximately the same proportion as for the β-galactosides and β-glucosides which have similar ring configurations. The α-galactosidase activity decreases, how-

ever, and in the same ratio as that for the β-L-arabinosidase activity. Additional support for ascribing the hydrolysis of the α-arabinosides to β-galactosidase and of β-xylosides to β-glucosidase is given by the similar effects of structural changes in the aglycon of the corresponding glycosides. Thus, the substitution of a methyl group in the ortho position of phenyl β-glucoside, β-galactoside, β-xyloside, and α-L-arabinoside increases the ease of hydrolysis about 15 times in all cases (24). For the hydrolysis of

TABLE 1

INFLUENCE OF PURIFICATION OF ALMOND EMULSIN
ON HYDROLYSIS OF SEVERAL GLYCOSIDES

Substrate	Enzyme efficiency		Ratio (I:II)	Ref. No.
	Rohferment (I)	Silver-purified emulsin (II)		
Phenyl β-D-glucoside	0.33	2.55	1:7.7	9
Phenyl β-D-galactoside	0.046–0.049	......	....	9
	0.032	0.24	1:7.5	55
Phenyl α-D-galactoside	0.0032	......	....	55
	(9.6)*	(0.5)*	1:0.05	39
Phenyl α-D-mannoside	0.10	0.17	1:1.7	55
Phenyl α-L-arabinoside	0.022	0.17	1:7.7	24
	(32)*	(250)*	1:7.8	39
Phenyl β-L-arabinoside	(7)*	(0.6)*	1:0.09	39
Phenyl β-D-xyloside	0.0018	0.017	1:9.4	9, 24, 55
Phenyl α-D-lyxoside	(0.00094)**	......	....	50

* The values given were not measured under standard conditions and cannot be directly compared, in absolute value, with the others.

** Because of the insolubility of the lyxoside, the concentrations used were more dilute than usual.

α-lyxosides, the enzyme should be α-mannosidase; this is supported by the stability to heat of the responsible enzyme. In this instance, however, the glycoside is derived from a non-naturally occurring sugar for which it seems highly improbable that a special enzyme, *i. e.*, one hydrolyzing *only lyxosides*, would be found. It seems probable, therefore, that the hydrolysis is to be ascribed to the α-mannosidase which acts on the structurally related hexosides (α-mannosides) (50).

For some purposes, the absence of certain enzymes in almond emulsin is of importance. Thus, the absence of α-glucosidase provides a basis for determining whether an unknown compound is an α- or a β-glucoside.*

* Note that this distinction holds only for the *glucosides* and not for glycosides in general, as is sometimes claimed.

In order to demonstrate that a given enzyme is not present, it is necessary to use as drastic conditions as possible. Unless highly purified enzymes are used, values of the enzyme efficiency (*Wertigkeit*) of less than about 10^{-5} are usually not significantly greater than the experimental error. For this reason, it has been suggested that "unhydrolyzability" be defined as an enzyme efficiency of less than 10^{-5}, that is—an enzyme is said (58) not to be present if the measured activity on a phenyl glycoside is less than 10^{-5}. The phenyl or some more easily hydrolyzed glycoside is used as the test substrate, since the rates are influenced by the nature of the aglycon groups.

Table II lists some enzymes which are absent from almond emulsin, or nearly so, and gives the maximum activities observed.

TABLE II

MAXIMUM VALUES FOR ACTIVITY OF CERTAIN GLYCOSIDASES ("ROHFERMENT")

Enzyme	Substrate	Enzyme efficiency (*Wertigkeit*), maximum value	Ref. No.
α-D-Glucosidase	Phenyl α-D-glucoside	7×10^{-6}	10
	Maltose	5×10^{-5}	49
α-L-Mannosidase	Phenyl α-L-rhamnoside	5×10^{-7}	10
β-L-Mannosidase	Phenyl β-L-rhamnoside	3×10^{-7}	10
α-D-Arabinosidase (β-L-Galactosidase)	Phenyl α-D-arabinoside	1×10^{-6}	10
β-D-Arabinosidase (α-L-Galactosidase)	Phenyl β-D-arabinoside	3×10^{-7}	10
Fructopyranosidase	Phenyl β-D-fructopyranoside	1×10^{-4}	10
Fructofuranosidase (invertase)	Sucrose	$(2 \times 10^{-4})^*$	53
β-D-Glucosaminidase	Phenyl β-D-glucosaminide	Nil	23
β-D-Mannosidase	Phenyl β-D-mannoside	Nil	10

* Enzyme value for 0.13 M substrate solution.

3. Specificity of β-Glucosidase

Influence of Substitution and of Changes in the Configuration of the Sugar Component of β-Glucosides.—Several β-glucosides have been prepared in which the hydroxyls on carbons 2, 3, and 4 have been substituted by methoxyl and p-toluenesulfonyl (tosyl) groups. All such derivatives have been found "unhydrolyzable" by almond emulsin. Thus, the 2-tosyl, 3-tosyl, and 4-tosyl derivatives of vanillin β-glucoside

all exhibit only insignificant hydrolysis after more than 100 hours (15). The 3-methyl, and the 2,4,6-trimethyl derivatives of phenyl β-glucoside are also not affected by almond emulsin (25, 58).

It would be of interest to study the hydrolyzability of the 2-methyl and 4-methyl derivatives of phenyl β-glucoside, since these derivatives have small groups substituted on the opposite side of the pyranose ring from that on which the 3-methyl group is located. If the compounds are hydrolyzable, this would provide evidence that the enzyme is attached to the opposite side of the ring.

When the hydroxyl of carbon 2 of glucosides (or mannosides) is replaced by a hydrogen to give a 2-desoxyglucoside, a glycoside is produced which is closely related to both glucosides and mannosides. The phenyl 2-desoxy-α-D-glucoside is hydrolyzed by almond emulsin (E.E. = 0.00029); and, as is evidenced from the stability of the enzyme to heat, the enzyme responsible is α-mannosidase (22). It would be presumed that the beta isomer might be hydrolyzed by β-glucosidase. This type of change in the pyranose ring is the only type known which does not result in a complete loss of hydrolyzability by glycosidases. Presumably, this is due to the lack of bulk of the substituent and to its negligible influence on the general conformation of the pyranose ring.

Substitutions at carbon 6 of β-D-glucosides affect the enzymatic action differently from those at the 2, 3, and 4 positions. In the accompanying formula, the substitutions under consideration are indicated by the group X.
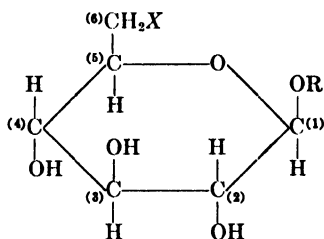
Some values found (16) for the enzyme efficiencies (*Wertigkeit*) of several such compounds are given in Table III.

By plotting the volumes of the substituent groups against the logarithms of the corresponding enzyme efficiencies (*Wertigkeiten*), Helferich, Grünler, and Gnüchtel (16) were able to show a linear relationship between these two quantities. It would seem then that *substitutions at carbon 6 have only a quantitative influence on the rate of hydrolysis* of the β-glucoside by almond

emulsin. Only when the group becomes too large does the rate of hydrolysis become inappreciable. Thus, *o*-hydroxymethylphenyl 6-benzoyl-β-glucoside (populin) and vanillin 6-tosyl-β-glucoside exhibit little or no hydrolysis when subjected to the action of almond emulsin for long periods, although the corresponding substances with unsubstituted 6-hydroxyls (salicin and vanillin glucoside) are very easily hydrolyzed in several minutes under the same conditions (15, 58).

TABLE III

INFLUENCE OF SUBSTITUTION AT CARBON 6 ON THE ENZYMATIC
HYDROLYSIS OF β-GLUCOSIDES

Substituent at carbon 6 (group X)	Enzyme efficiency for phenyl β-(6-X)-glucoside	Ease of hydrolysis* of the 6-substituted vanillin β-glucoside	Volume of group X† (Biltz), ml.
H	0.56	...	5.8
OH	0.3	39	9.4
F	0.03	6**	15.9
Cl	....	2.5	16.5
Br	0.003	1.5	19.5
OCH₃	0.0023	...	24.4
I	....	0.17	24.3

* These values are calculated in the same manner as for the enzyme efficiencies but, because of the limited solubilities of the substrates, 0.00104 M solutions were employed instead of the usual 0.052 M solutions.
** Extrapolated value.
† Molecular volumes.

Presumably, the slow rate of hydrolysis (23) of phenyl 6-amino-β-glucoside (E.E. $= 3 \times 10^{-5}$) and the slightly greater rate of hydrolysis of phenyl 6-acetylamino-β-glucoside (E.E. $= 5.8 \times 10^{-4}$) is to be ascribed to the ionic charge of the amino group.

Another type of substitution at carbon 6 is represented by the pentoses, hexoses, and heptoses of the corresponding ring types. Thus, L-arabinosides, D-galactosides, and the D-α-mannoheptosides (also known as D-manno-D-gala-heptosides and D-*glycero*-D-*galacto*-aldoheptosides) have the same pyranose ring and differ merely in having H, —CH₂OH and —CHOH-—CH₂OH groups substituted at carbon 5. D-Xylose and D-glucose, as well as D-lyxose and D-mannose, are related in a similar manner, as illustrated in the accompanying formulas.

The evidence for believing that the pentosides are hydrolyzed by the same enzymes as those hydrolyzing the corresponding hexoside types has

been previously discussed (page 46). Additional work (51) of interest in this connection is the study of the hydrolyzability of the above-mentioned heptosides, derived from mannose but having the same conformation for the pyranose ring as the D-galactosides. As shown in the above formulas, these compounds are of the β- and α-D-galactoside ring types. Inasmuch as almond emulsin contains both α-galactosidase and β-galactosidase, it would be expected that both of the phenyl glycosides (α and β) of this heptose sugar should be hydrolyzed by almond emulsin, since the

X

OH ⟋ C————O OR
H C
H OH H H
C————C
H OH
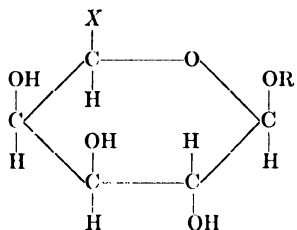
β-D-GALACTOSIDE TYPE:
α-L-arabinoside (X = H)
β-D-galactoside (X = CH₂OH)
β-D-α-mannoheptoside (X = —CHOH—CH₂OH)
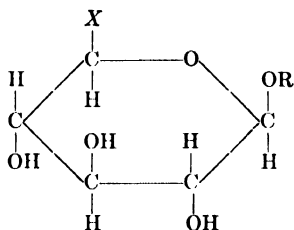
X

H ⟋ C————O OR
C H C
OH OH H H
C————C
H OH

β-D-GLUCOSIDE TYPE:
β-D-xyloside (X = H)
β-D-glucoside (X = CH₂OH)

X

H ⟋ C————O H
C H C
OH OH OH OR
C————C
H H
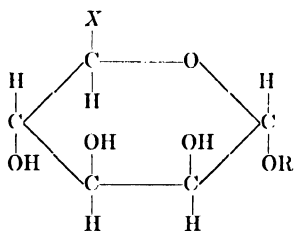
α-D-MANNOSIDE TYPE:

α-D-lyxoside (X = H)

α-D-mannoside (X = CH₂OH)

compounds are D-galactosides substituted at carbon 6. However, since the substituent groups are fairly large, the rate would be expected to be rather small. Actually, it was found that the beta isomer was slowly hydrolyzed, but that the alpha isomer was not appreciably affected. The lack of hydrolysis of the alpha isomer is probably due to the smaller amount of α-galactosidase as compared with the β-galactosidase, in almond emulsin. It is of interest to compare the relative rates of hydrolysis of compounds related to several hexose types. This is done in Table IV.

The effect of configurational changes in the pyranose ring of hydrolyzable hexose types has also received a little investigation. Such changes result in the formation of new hexose types; and when *all* of the asymmetric carbons are involved, such changes result in a shift from the D- to the L-series or from the L- to the D-series. The L-glucosides were reported by Fischer (6) to be unhydrolyzed by enzymes, but this work requires confirmation.

TABLE IV

COMPARISON OF EASE OF HYDROLYSIS OF PENTOSIDES, HEXOSIDES, AND HEPTOSIDES OF SIMILAR RING TYPES

Substrate	Enzyme efficiency	•Relative ease of hydrolysis (hexoside = 100)
β-D-Galactoside type		
Phenyl α-L-arabinoside	0.022	69
Phenyl β-D-galactoside	0.032	100
Phenyl β-D-α-mannoheptoside	0.00022	0.7
α-D-Galactoside type		
Phenyl α-D-galactoside	0.0032	100
Phenyl α-D-α-mannoheptoside	$<10^{-5}$	<0.3
β-D-Glucoside type		
Phenyl β-D-xyloside	0.0018	0.55
Phenyl β-D-glucoside	0.33	100
α-D-Mannoside type		
Phenyl α-D-lyxoside	0.00094	0.9
Phenyl α-D-mannoside	0.10	100

A comparison of the action of almond emulsin on phenyl-α-mannoside and phenyl-α-taloside is of interest because these substances differ only in the configuration of carbon 4. Although the mannoside is easily cleaved, the taloside is not affected (51). Similarly the methyl α- and β-D-gulosides are not hydrolyzed (31), although they differ from the hydrolyzable galactosides only in the configuration of carbon 3.

The phenyl α- and β-D-α-glucoheptosides, which are of the L-gulose type, also exhibit no appreciable hydrolysis by almond emulsin.

The above "unhydrolyzable" glycosides are all derivatives of sugars which have never been found in natural products. It might be expected that special enzymes, *i. e.*, enzymes hydrolyzing only these compounds, would be absent. The hydrolyzability of the glycosides of the lyxose and β-D-α-mannoheptose, which are synthetic sugars having the pyranose rings of naturally occurring sugars (mannose and galactose), makes it probable that such rings are a necessary prerequisite for hydrolysis. This condition may be summarized as: *Glycosides with unsubstituted rings having the same conformation as those in a naturally occurring sugar may be expected to be hydrolyzed by enzymes;* and, conversely, *naturally occurring enzymes will hydrolyze only naturally occurring hexoside types.*

Aglycon Specificity.— *Alkyl glucosides.*—Many alkyl β-glucosides have been prepared and tested for their ease of enzymatic hydrolysis by Helferich, Veibel, and their associates and more recently by Pigman and Richtmyer. Many of the results are summarized in Figure 2.

The figure, originally published by Pigman and Richtmyer (57), summarizes their data as well as those of Helferich and of Veibel and Lillelund (64, 65). It has been revised to include more recent data of Helferich and Goerdeler (13). Veibel and associates have studied extensively the kinetics of the hydrolysis, including the effect of substrate concentration. By interpolation of these results at the standard concentration for such measurements (0.052 *M*), it has been possible to calculate comparable values. In the subsequent discussion this has been done unless otherwise indicated.

In the *n*-alkyl series, the hydrolysis of β-glucosides with aglycon groups up to nine carbon atoms in length (methyl to nonyl) have been studied (57). When the carbon chains are longer than nine atoms, the glucosides become too insoluble for study at the usual concentrations. As shown in Figure 2, there is a progressive increase in the ease of hydrolysis as the carbon chain is lengthened. Other homologous series, such as the cyclohexyl and benzyl series, exhibit a similar correlation. In the primary alcohol series, however, there is an optimal length for the aglycon group at 7 to 8 carbons; and at greater chain lengths the ease of hydrolysis decreases.

It has been suggested that the existence of the maximum for the β-glucosides of the primary alcohol series may be due to an increase in the number of adsorption bonds between the aglycon group and the enzyme as the number of atoms in the chain increases. Although this would tend to facilitate the hydrolysis, the rate would not increase indefinitely but instead, at some point, the progressively decreasing dissociation of the products of hydrolysis from the enzyme would act to inhibit the over-all reaction. That is, the rate-determining reaction would be the dissociation

of the products of hydrolysis and neither the cleavage of the glucosidic
linkage nor the adsorption (57).

This interpretation of the influence of an increase in the chain length
of the aglycon as being due to an increased adsorption of the aglycon group
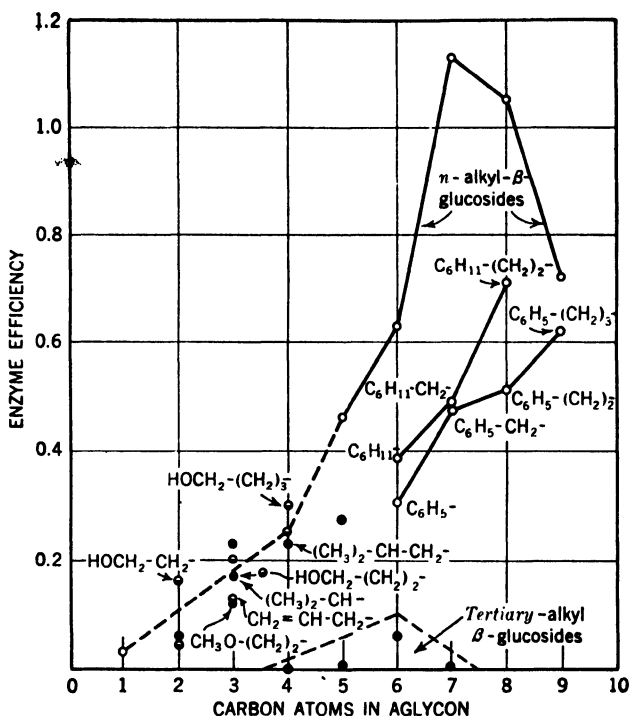
Fig. 2.—Comparison of the ease of hydrolysis (E.E.) of alkyl β-glucosides by sweet-
almond β-glucosidase with the number of carbon atoms in the aglycon (alkyl) groups.

Open circles represent data of Pigman and Richtmyer (57) and filled circles those of
Veibel and Lillelund (64, 65). The half-filled circles indicate data of Helferich and
associates (13). Circles for which formulas are not given represent members of the
n-alkyl β-glucoside series.

receives support from measurements of the dissociation constants of the
enzyme–substrate compounds. Veibel and Lillelund (65) have deter-
mined these constants for a number of alkyl β-glucosides and their results
are compared in Figure 3 with the number of carbons in the aglycon
groups. It will be seen that compounds with straight-chain aglycons

are adsorbed the most easily (smallest dissociation constants) and that the association increases as the chain length becomes greater. The straight chains are more highly adsorbed than the branched-chain compounds as might be expected, since the former should be more able to accommodate themselves to the active areas on the enzyme surface and to cover a larger portion of the active areas.
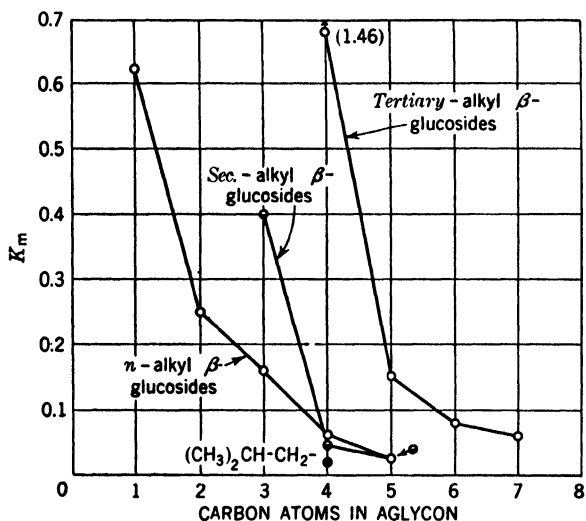
Fig. 3.—Effect of the structure of the alkyl groups of alkyl β-glucosides on the Michaelis dissociation constant (K_m) for the enzyme–substrate compound.

If the influence of structural changes in the aglycon group is due to direct effects on the glucosidic linkage undergoing hydrolysis, a close correlation between the rates of the acid and of the enzyme-catalyzed reactions might be expected. Several investigations have been carried out in which this relationship was tested; but, in general, it has been shown that no such correlation exists. The subject recently has been reinvestigated by Veibel and Frederiksen (63). Some of their results are reproduced in Table V.

The lack of correlation of the ease of enzymatic and acid hydrolysis becomes particularly apparent when the values for the *tert*-alkyl glucosides are compared with the others. The former are easily hydrolyzed by acids, even at room temperature, although they are very difficultly hydrolyzed

W. W. PIGMAN

by β-glucosidase. For the enzymatic hydrolysis, the activation energies of the *tert*-alkyl glucosides are considerably larger than those for the others, although there is little difference between the values for the acid-catalyzed reactions. The writer has observed similar differences (unpublished) between the acid and enzymatic hydrolysis of alkyl and aryl β-glucosides. Although, as previously shown, the ease of enzymatic hydrolysis of the *n*-alkyl β-glucosides increases with chain length until the chain is seven to

TABLE V

COMPARISON OF ACID AND ENZYMATIC HYDROLYSIS
OF SOME ALKYL β-GLUCOSIDES

Structure of aglycon group	Acid hydrolysis ($N/1$ HCl)			Enzyme hydrolysis at 30° C.			
	$k \times 10^5$, 68° C.	Q*	B*	$k'_{obs.} \times 10^3$	$k_3 \times 10^3$**	Q*	B*
CH₃—	0.35	32,610	16.0	1.0	0.73	12,200	5.7
CH₃·CH₂·CH₂—	0.46	32,430	16.0	8.7	1.95	13,500	7.0
(CH₃)₂·CH—	0.79	32,090	16.0	6.5	2.88	13,100	6.9
(C₂H₅)₂·CH—	1.18	31,520	15.8	84.0	5.10	10,600	5.3
(CH₃)₃·C—	9.97$^{40°}$	30,810	17.5	0.011	0.02	19,960	9.7
(CH₃)₂·C(C₂H₅)—	42.9$^{40°}$	29,870	17.5	0.22	0.042	19,960	10.0

* $\ln k = (-Q/RT) + B$.

** k_3 is calculated from the observed velocity constant $k'_{obs.}$, and is said to give the rate of hydrolysis of various glucosides for the same degree of dissociation of the enzyme-substrate complex. It shows a somewhat different order of constants from the corresponding observed reaction constants and enzyme efficiencies which do not take this factor into consideration. The calculation is made on the basis of the relation: $k_3 = k'_{obs.} (K_m + c)$, where K_m is the Michaelis dissociation constant, c is the substrate concentration, and $k'_{obs.}$ is the observed velocity constant calculated to standard conditions. All velocity constants are expressed with seconds and natural logarithms as the basis. The $k'_{obs.}$ values were recalculated to the above basis and are for 0.04 M substrate solutions.

eight carbons long, the rates of acid hydrolysis of these compounds show very little difference. Even more striking is the series with aglycon groups represented as C₆H₅·(CH₂)ₙ—. In this case also, the ease of enzymatic cleavage increases with *n*, but the ease of acid hydrolysis decreases with increase of *n* and is greatest for the first member of the series (phenyl β-glucoside).

The enzyme efficiencies of other alkyl β-glucosides and derivatives are given in Table VI (13, 19, 27, 35, 57).

At the present stage of progress, it is difficult to interpret these effects of structural variations. For aglycons of the structure X·CH₂·CH₂—.

the order of several groups (X) in promoting the enzymatic hydrolysis is $I > Br > C_6H_5, SO_2C_2H_5, Cl > CH_3 > OH > H$. Since, as shown (56) by the optical rotations of these glucosides, the effects of these groups are not transmitted through the carbon chain to the glucosidic linkage undergoing hydrolysis, it seems that the observed effects are due to differences in the adsorption of the aglycon group by the enzyme. It is interesting that the iodine atom is almost as effective as four methylene groups (E.E. for $I \cdot CH_2 \cdot CH_2 \cdot O \cdot Gl$ is 0.95 as compared with 1.13 for the n-hexyl glucoside). Nearly all the homologous series exhibit an increase in the ease of hydrolysis with increasing chain length.

TABLE VI

EASE OF HYDROLYSIS OF SOME SUBSTITUTED ALKYL β-GLUCOSIDES

β-Glucoside	Enzyme efficiency. $X =$						
	—CH$_3$	OH	Cl	I	—SO$_3$H	—SO$_2$C$_2$H$_5$	C$_6$H$_5$
X—(CH$_2$)$_2$—O—Gl	0.19	0.16	0.44	0.94	0.0009	0.32	0.51
X—(CH$_2$)$_3$—O—Gl	0.25	0.17	0.53	0.44	0.08	0.8	0.62
X—(CH$_2$)$_4$—O—Gl	0.47	0.30	0.63	1.1	0.36	1.2	..

Aglycon group	Enzyme efficiency
CH$_3$—	0.036
CH$_3 \cdot$CH$_2$—	0.045
CH$_2$Br$\cdot$CH$_2$—	0.58
CH$_2$:CH$\cdot$CH$_2$—	0.13
CH$_2$:CH$\cdot$CH(CH$_3$)—	0.51
CH$_2$:CBr$\cdot$CH$_2$—	0.74
CH$_2$Br:CHBr$\cdot$CH$_2$—	1.7
NH$_2 \cdot$CO$\cdot$NH$\cdot$N:CH$\cdot$CH$_2$—	0.77

Glucosides having sugars as aglycons (disaccharides) are of importance because there are some claims that the disaccharides (holosides) are not hydrolyzed by the same enzymes as those hydrolyzing the ordinary glycosides (heterosides). All the disaccharides with β-glucosidic or β-galactosidic linkages which have been tested are hydrolyzable by almond emulsin. In Table VII, the ease of hydrolysis of several disaccharides and related compounds are compared. For the origin of the data listed see references 14, 49, and 52.

The pronounced effect of even slight changes in the aglycon groups is very evident. Thus, cellobiose (4-glucose β-glucoside) and 4-mannose β-glucoside differ only in the configuration of a single carbon in the agly-

cons, carbon 2; and yet this change from the glucose to the mannose configuration produces a very marked influence in the ease of hydrolysis. Similarly, 4-mannose β-glucoside and celtrobiose (4-altrose β-glucoside) differ only in the configuration of carbon 3 of the aglycon groups; and yet the ease of hydrolysis is in the ratio of 1:10. A change in the position of the glucosidic linkage from carbon 4 (cellobiose) to carbon 6 (gentiobiose) of the aglycon sugar with no other changes in structure or configuration reduces the rate of hydrolysis about one-half. For the β-galactosides,

TABLE VII

EASE OF ENZYMATIC HYDROLYSIS OF DISACCHARIDES AND RELATED COMPOUNDS
WITH β-GLUCOSIDIC AND β-GALACTOSIDIC LINKAGES

β-Glucosides			β-Galactosides		
Substrate	Structure	E.E. × 10³	Substrate	Structure	E.E. × 10³
Cellobiose	4-Glucose β-glucoside	159, 180	Lactose	4-Glucose β-galactoside	11.2
Ge. tiobiose	6-Glucose β-glucoside	75	Lactulose	4-Fructose β-galactoside	(14)*
Celtrobiose	4-Altrose β-glucoside	(23)*	Neolactose	4-Altrose β-galactoside	(2.8)*
	4-Mannose β-glucoside	2.3	Lactositol	4-Sorbitol β-galactoside	0.84
Phenyl α-cellobioside	4-(Phenyl α-glucoside) β-glucoside	160	Lactobionic acid	4-(Gluconic acid) β-galactoside	0.41
Phenyl β-glucoside		330	Phenyl β-lactoside	4-(Phenyl β-glucoside) β-galactoside	23
			Protocatechuic aldehyde β-lactoside	4-(Protocatechuic aldehyde β-glucoside) β-galactoside	80
			Phenyl β-galactoside		32–49

*Not measured originally under standard conditions. The values given were calculated from the corresponding values for lactose or cellobiose, measured under the same conditions, and are approximately those to be expected under the standard conditions. The data for celtrobiose were derived from the published data of Richtmyer and Hudson, *J. Am. Chem. Soc.*, **61**, 1834 (1939).

corresponding differences may be observed. The effect of reducing the aglycon group of lactose to a sorbitol and of oxidation to a gluconic acid group is shown by the values for lactositol and lactobionic acid of 0.84 × 10^{-3} and 0.41 × 10^{-3}, respectively.

The marked influence of configurational changes in the aglycon sugars of these disaccharides might be explained on the basis of their effect on the adsorption of the aglycon on the enzyme. Since, as previously mentioned, the hydroxyl groups are probably more easily adsorbed than hydrogens, the relative distribution of these groups would be important. The

change in configuration of a single asymmetric center would undoubtedly influence the number of adsorption bonds which the aglycon group could form with the enzyme. The effect, however, would be much greater than the loss or gain of a single linkage, since the change of configuration of one carbon simultaneously affects the conformation of the entire aglycon group.

TABLE VIII

EFFECT OF SUBSTITUTIONS IN THE AROMATIC NUCLEUS ON THE HYDROLYSIS OF PHENYL β-GLUCOSIDE BY SWEET-ALMOND β-GLUCOSIDASE

Monosubstituted				*Disubstituted*	
Group X	Enzyme efficiency			Groups and position	Enzyme efficiency
	Ortho subst.*	Meta subst.**	Para subst.†		
None (H)	0.33	0.33	0.33	p-CHO (mono)	4.2
CHO	8.6	..	4.2	p-CHO, o-OH	9.7
CH₃—	4.3	0.55	0.12	p-CHO, o-OCH₃	13
CH₃O—	3.5	..	0.27	p-CHO, o-C₆H₅·CH₂O—	2.3
CH₃·CO—	3.3	..	1.1	p-CH₃·CH:CH—, o-OCH₃—	1.1
CH₃·CH₂—	2.3	..	0.08	3-CHO, 6-OCH₃	11
CN·CH₂—	2.0	..	1.2	3-CHO, 6-OC₂H₅—	2.5
HO·CH₂—	2.0	..	...	o-CH₃, o'-CH₃—	0.1
CH₃O·CO—	1.6	1.5	1.5	o-CH₃, p-CH₃—	1.4
HO·CO—	1.6	0.60	0.43	o-CH₂OH (mono)	2.0
Ac·NH·CH₂—	0.88	1.48	0.13	o-CH₂OH, p-Cl	0.48
HO—	0.56	0.47	0.059	o-CH₂OH, p-Br	0.60
HO·CO·CH₂—	0.13	..	0.64	o-CH₂OH, p-I	(0.62)
NH₂—	...	..	0.055		
NO₂—	...	..	0.53		
NH₂·CH₂—	0.036	0.05	0.027		
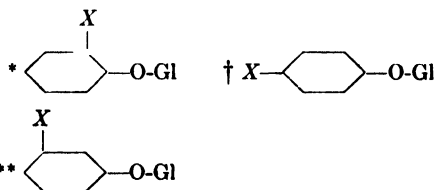
Aryl β-glucosides.—The rate of enzymatic hydrolysis of a great many substituted-phenyl β-glucosides has been studied in particular by Helferich and associates (12, 26, 28, 29, 32, 34, 52). The results are summarized in Table VIII.

On the basis of these data, the following generalities on the effect of sub-

stitution in the benzene nucleus on the rate of enzymatic hydrolysis are given, with phenyl β-glucoside taken as the basis for the comparison.

1. The introduction of the so-called "meta-directing groups" into any position tends to increase the rate of hydrolysis; aldehyde groups are particularly effective.

2. The introduction of an amino group always lowers the rate of hydrolysis; but acetylation of the amino group counteracts this decrease.

3. When any group but the amino group is substituted in the ortho position, the rate of hydrolysis is increased considerably.

4. "Meta-directing groups" in the para position increase the ease of hydrolysis but to much less an extent than when in the ortho position. Other groups decrease the rate of hydrolysis.

5. Groups in the meta position produce effects intermediate between those observed for the ortho and para positions; however, in general, they seem to increase the rate of hydrolysis.

6. When two groups are substituted in the aromatic nucleus, the influences are more or less additive. Since ortho effects are greater than para effects (which may also be inhibiting), a group such as methyl, when in both positions, increases the rate over that for the phenyl glucoside; but the rate is less than that for the corresponding monoortho-substituted derivative.

It should be noted that the results apply only to the β-glucosidase and probably the β-galactosidase of almond emulsin. As will be shown later, similar enzymes from other sources exhibit quite different specificities.

It is difficult to interpret these results at our present stage of knowledge. In general, it may be stated that the effects observed are not due to influences transmitted through the ring. Attempts by the writer to correlate the results with the "sigma values" of Hammett and with the optical rotations of the glucosides have been unsuccessful. This is also illustrated by the similar behavior of groups separated from the aromatic ring by a methylene group as compared with the behavior of the same groups when attached directly to the aromatic nucleus. If the influence of these groups could be ascribed to their effect on the glucosidic linkage undergoing hydrolysis, it might also be expected that there would be a parallelism between the rate of enzymatic and acid hydrolysis; but, as shown (33, 34) from the few results available (Table IX), such is not the case. Thus, the phenyl glucoside is hydrolyzed by acids much more rapidly than the p-hydroxyacetophenone β-glucoside; but the latter is hydrolyzed about three times more rapidly by enzymes than is the phenyl glucoside.

Since the observed differences are undoubtedly due to several influences,

including resonance and inductive effects, on the glucosidic linkage as well as those on the adsorption of the aglycon group, it is difficult to isolate the different causative factors. At present, however, the most probable explanation for the actions of the same groups in the o-, m-, and p-positions would seem to be the formation of weak bonds between these groups and groups in the enzyme surface. It would be expected that groups in the

TABLE IX

COMPARISON OF THE RATES OF ACID AND ENZYME HYDROLYSIS
OF SOME AROMATIC β-GLUCOSIDES

Aglycon group	Enzyme efficiency	Acid hydrolysis, $k \times 10^4$
C_6H_5—	0.34	23
o-$CN \cdot CH_2 \cdot C_6H_4$—	2.0	4.9
p-$CN \cdot CH_2 \cdot C_6H_4$—	1.2	16
o-$CH_3 \cdot CO \cdot C_6H_4$—	3.3	110
p-$CH_3 \cdot CO \cdot C_6H_4$—	1.1	8
o-$CHO \cdot C_6H_4$—	8.6	9
p-$CHO \cdot C_6H_4$—	4.2	8
o-OH, p-$CHO \cdot C_6H_3$—	10	13
6-OCH_3, 3-$CHO \cdot C_6H_3$—	11	43
o-OCH_3, p-$CHO \cdot C_6H_3$—	13	35
p-CH_3—	0.12	21
o-CH_3—	4.3	18
o-CH_2OH—	1.9	11

ortho position close to the linkage undergoing hydrolysis would be more effective than those removed a carbon (meta position), and still more effective than those removed still another carbon atom (para position). The ionic amino group might form much more stable bonds, which would favor hydrolysis but which would slow up the over-all reaction because of the slow dissociation of the products of hydrolysis from the enzyme.

IV. Specificities of Glycosidases from Various Sources

Seeds, animal tissues and organs, and microorganisms are particularly rich sources of enzymes; and emulsins* containing glycosidases may be frequently obtained by aqueous extraction of these materials. It has often been assumed that the enzymes hydrolyzing the same substrates but from

---

* As explained in more detail on page 72 et seq., the term "emulsin" is used in a general sense as a synonym for "crude or partially purified mixtures of enzymes" from any source.

different sources are identical, *i. e.*, that the β-glucosidase of almond emulsin is identical with that in *Aspergillus oryzae* emulsin (takadiastase) and in emulsins from animal sources. Although the early work of Fischer (7) showed that the α-galactosidases of yeast and of animal organs are different, Weidenhagen later assumed that they are identical. Much of the available evidence concerning the identity or lack of identity of enzymes with similar action will be considered in the present section.

1. β-Glucosidases

A very important study of the distribution and specificities of β-glucosidases has been carried out by Miwa, Cheng, Fujisaki, and Toishi (47). Their results are summarized in Table X.

TABLE X

COMPARISON OF SPECIFICITIES OF β-GLUCOSIDASES FROM DIFFERENT SOURCES

Source of emulsin	β-Glucoside							
	Phenyl		Salicyl		o-Cresyl		p-Cresyl	
	f*	Ratio**	*f**	Ratio**	*f**	Ratio**	*f**	Ratio**
Prunus armeniaca (apricot)	2.47	1.0	30.7	12.5	59.8	24.2	1.27	0.51
Amygdalus communis (sweet almond)	2.10	1.0	29.1	13.9	52.5	25.0	1.20	0.57
Prunus persica (peach)	0.315	1.0	3.16	10.0	5.84	18.5	.169	0.53
Cycas revoluta (sago palm)	0.0226	1.0	0.176	7.65	0.394	17.4	0.0114	0.50
Papaver somniferum (opium poppy)	0.00459	1.0	0.0163	3.54	0.0268	5.82	0.00411	0.89
Glycine hispida (soybean)	0.00073	1.0	0.00181	2.48	0.00071	0.97	.00146	2.0
Cucurbita moschata (squash)	0.00913	1.0	0.00503	0.55	0.00421	0.46	.00897	1.00
Aspergillus oryzae (takadiastase)	0.110	1.0	0.0962	0.88	0.0139	0.13	0.0722	0.59
A. oryzae	1.058	1.0	0.843	0.80	0.013	0.12	0.749	0.71
A. niger	2.99	1.0	0.965	0.32	0.0772	0.025	4.34	1.46
Ergot	0.148	1.0	0.0523	0.35	0.0349	0.24	0.148	1.0

* "*f*" is a measure of the ease of hydrolysis similar to the enzyme value and enzyme efficiency, but 0.012 *M* substrate solutions have been employed and the enzyme concentration is expressed as g. in 8 ml.

** For each enzyme preparation, the ease of hydrolysis of the phenyl glucoside is taken as unity.

The great difference in the ease of hydrolysis of ortho and para isomers, originally discovered by Helferich and associates, has been used frequently to characterize enzymes from different sources. The β-glucosidases derived from the first four sources of Table X have identical specificities and exhibit a pronounced ortho substitution effect. The fungal β-glucosidases, however, appear to be quite different; and ortho substitution in the aglycon group reduces the ease of hydrolysis. Those from other

plant sources seem to be intermediate in type, and to be much less affected by substitution in the aglycon group.

The β-glucosidases of almond emulsin and of snail emulsin, made from the digestive juices of the snail (*Helix pomatia*), have also been compared (13). The results are summarized in Table XI.

The β-glucosidase found in the snail digestive juices is different from that of almond emulsin and shows much less effect of substitution in the aglycon group on the rate of hydrolysis; but the two are similar in that ortho substitution increases, and para substitution decreases, the rate.

TABLE XI

COMPARISON OF SPECIFICITIES OF β-GLUCOSIDASES OF ALMOND EMULSIN
AND OF SNAIL EMULSIN

| β-Glucoside of | Sweet-almond emulsin | | Snail emulsin | |
	E.E.	Ratio*	E.E.	Ratio*
Phenol	0.33	1.0	0.028	1.0
Salicyl alcohol	1.7	5.2	0.034	1.2
Vanillin	13	39	0.151	5.4
p-Cresol	0.12	0.36	0.026	0.9
o-Cresol	4.3	13	0.035	1.3
Ethanol	0.045	0.14	0.0056	0.2

* For each enzyme preparation, the E.E. for phenyl glucoside is taken as unity.

Highly purified yeast invertase preparations have a slight activity for the hydrolysis of β-glucosides which is probably due to the presence of small quantities of a β-glucosidase (1). In contrast to almond emulsin, which hydrolyzes cellobiose much more rapidly than gentiobiose, the yeast β-glucosidase hydrolyzes gentiobiose at an appreciable rate, although cellobiose is not affected under the experimental conditions. Amygdalin, *d*-mandelic-nitrile β-gentiobioside, is hydrolyzed more easily than gentiobiose itself. Phenyl β-glucoside is cleaved at about the same rate as gentiobiose, although for sweet-almond β-glucosidase the rate of hydrolysis of phenyl β-glucoside is much greater than that of gentiobiose.

Yeast α-glucosidase preparations also contain a β-glucosidase (49). The hydrolysis of cellobiose by this enzyme, in contrast to that in yeast invertase, is probably to be ascribed to the larger amounts present in the α-glucosidase preparation. The presence of this enzyme in such preparations requires that caution be exercised in assigning an alpha configuration to glucosides which are hydrolyzed by yeast α-glucosidase emulsins. How-

ever, the stability of the yeast β-glucosidase is in contrast to the instability of yeast α-glucosidase and may be used for ascribing the hydrolysis of a glucoside to one or the other of the yeast emulsin components.

The ability of emulsins from various sources to hydrolyze salicin (o-hydroxymethylphenyl β-glucoside) and phlorizin has been studied by Hofmann (42). Almond emulsin hydrolyzes salicin rapidly but phlorizin only very slowly. However, a preparation from horse kidneys cleaves the latter more easily than either salicin or phenyl β-glucoside. In contrast, emulsins made from pig and cattle kidneys, as well as from horse liver and horse, cattle, and pig intestinal mucosa, resemble almond emulsin in their action on these substrates.

2. β-Galactosidases

As previously mentioned, for almond emulsin the β-galactosidase exhibits the same specificity toward changes in the aglycon group as the

TABLE XII

COMPARISON OF SPECIFICITIES OF β-GALACTOSIDASES FROM ALMOND
AND ALFALFA EMULSINS

Substrate	Almond emulsin		Alfalfa emulsin	
	E.E.	E.E.** (relative)	E.E.	E.E.** (relative)
Phenyl β-galactoside	0.032–0.049 av. 0.040	1.0	0.17	1.0
o-Cresol β-galactoside	0.69	17	0.13	0.76
p-Cresol β-galactoside	0.02	0.5	0.14	0.82
Protocatechuic aldehyde β-galactoside	7.5	190	0.36	2.1
Vanillin β-galactoside	1.35	34	. . .	. . .
Phenyl β-lactoside*	0.023	0.6	0.029	0.17
Proctocatechuic aldehyde β-lactoside*	0.08	2.0	0.021	0.12
Lactose	0.009	0.2	0.004	0.024

* These compounds have two β-galactosidic linkages. Presumably, the hydrolysis of the disaccharide linkage takes place first; and, since the other linkage would be more rapidly hydrolyzed (compare values for the corresponding galactosides), the values given represent mainly the rate of hydrolysis of the disaccharide linkage.

** For each emulsin, the E.E. for the phenyl glucoside is taken as unity.

β-glucosidase with which it may be identical. In particular, it exhibits the same effect of ortho and para substitution as mentioned for the β-gluco-

sidase. Emulsins from other sources, however, contain other β-galactosidases with markedly different specificities. An emulsin made from alfalfa seeds (lucerne emulsin) contains a β-galactosidase which has received considerable study (40). The β-glucosidase activity of this material, however, is very slight. In Table XII, the ease of hydrolysis of a series of β-galactosides by almond and alfalfa emulsins are compared (14, 33, 34).

Coffee emulsin (37) resembles alfalfa emulsin in having only slight β-glucosidase activity and in having a β-galactosidase with a similar specificity. This β-galactosidase does not exhibit a marked influence of ortho substitution; and the enzyme efficiencies for the phenyl and vanillin β-galactosides are, respectively, 0.0029 and 0.0027. (Compare with values in Table XII.)

3. α-Galactosidases

Both almond emulsin and brewers' yeast contain α-galactosidases although bakers' yeast (top yeast) is inactive in this respect. Weidenhagen and Renner (70) have studied the relative abilities of α-galactosidases from several sources to hydrolyze melibiose (6-glucose α-galactoside) and phenyl α-galactoside. Their results are given in Table XIII.

TABLE XIII

RELATIVE EASE OF CLEAVAGE OF MELIBIOSE AND OF PHENYL α-GALACTOSIDE
BY ENZYMES FROM SEVERAL SOURCES

Emulsin source	Relative ease of hydrolysis (melibiose taken as unity for each comparison)
Bottom yeast	0.67
Sweet-almond	1.1
Bitter-almond	0.84
Barley malt	0.15
Aspergillus oryzae (takadiastase)	<0.1

Very highly purified preparations of invertase from bottom yeasts provide the most active source of this enzyme, as shown by Adams, Richtmyer, and Hudson (1). The ease of hydrolysis of phenyl and methyl α-galactoside as compared with melibiose is 1.23 and 0.033, respectively. As might be expected from the configurational relationship of the pyranose rings of α-D-galactosides and β-L-arabinosides (see previous discussion on page 51), phenyl β-L-arabinoside is also hydrolyzed, although at a very slow rate.

4. β-Fructofuranosidases (Invertases) and Their Relationship to the Inulases

The difficulties involved in the preparation of fructofuranosides with different aglycons have limited the studies of the specific action of β-fructofuranosidases to oligosaccharides which contain fructofuranoside residues. The most important of these are:

Sucrose, α-glucopyranoside $\xrightarrow{1\text{-}2'}$ β-D-fructofuranoside

Raffinose, α-galactopyranoside $\xrightarrow{1\text{-}6'}$ α-glucopyranoside $\xrightarrow{1'\text{-}2}$ β-fructofuranoside

Melezitose, α-glucopyranoside $\xrightarrow{1\text{-}3'}$ β-fructofuranoside $\xrightarrow{2'\text{-}1}$ α-glucopyranoside

Stachyose, galactopyranoside $\xrightarrow{1\text{-}6'}$ galactopyranoside $\xrightarrow{1'\text{-}4}$ glucopyranoside $\xrightarrow{1\text{-}2'}$ β-fructofuranoside

Inulin, β-fructofuranose $\xrightarrow{1\text{-}2'}$ (β-fructofuranoside)$_n$ $\xrightarrow{1'\text{-}2}$ β-fructofuranoside

It will be noted that all the oligosaccharides, excepting melezitose, have terminal unsubstituted fructofuranoside residues. All are known to be hydrolyzed by yeast invertase preparations, again with the exception of melezitose. The lack of cleavage of the latter compound might be expected because the furanose ring is not unsubstituted.

The hydrolysis of these compounds has been investigated by Adams, Richtmyer, and Hudson (1) using their highly purified invertase preparations. Their results are summarized in Table XIV.

TABLE XIV

ACTION OF HIGHLY PURIFIED YEAST INVERTASE PREPARATIONS
ON SOME OLIGOSACCHARIDES*

Enzyme Preparations	Sucrose		Raffinose		Inulin		Stachyose	
	E.V.	Ratio	E.V.	Ratio	E.V.	Ratio	E.V.	Ratio
Bakers' yeast-A	791	1.00	182	4.35	0.282	2,800	(167)	14.7
Brewers' yeast-A	885	1.00	103	8.59	0.0313	28,300	...	..
Brewers' yeast-C	884	1.00	111	7.96	0.0529	16,700	(85)	32.3

* These values were determined under the standard conditions of Weidenhagen, and are for 0.1388 M substrate solutions except in the case of stachyose, for which one-fifth the standard concentration was employed. The ratios given represent the fraction of the corresponding activity toward sucrose, and, in the case of stachyose, values for sucrose at the same dilution were used.

These results show conclusively either that several enzymes are responsible for the hydrolysis of these substrates or that the enzymes differ somewhat according to their origin. It will be noted that the brewers' yeast-A emulsin has only one-tenth the inulase activity of the bakers'

yeast emulsin although the invertase activities are not greatly different. For this reason, and because of the markedly different hydrogen-ion concentrations required at optimal activity, the authors concluded that the two enzymes are not identical. It was not possible for the authors to study the effect of purification on these various activities, although such studies probably would have provided more positive evidence for these conclusions.

A comparative study (53) of the invertase and inulase contents of emulsins from a number of different sources shows that the ratio of the two activities varies widely and is as high as 4.8:1. This compares with a ratio of 28,300:1 for the brewers' yeast, as given in the Table XIV. The inulase and invertase contents of these emulsins are summarized in Table XV.

TABLE XV

COMPARISON OF INVERTASE AND INULASE CONTENT OF EMULSINS
FROM SEVERAL SOURCES

Emulsin source	Invertase value (I) × 10⁴	Inulase value (II) × 10⁴	Ratio of I: II
Aspergillus			
oryzae No. 1	420	58	7.2
oryzae No. 2	120	0.24	500
oryzae No. 3	320	1.50	210
niger No. 1	950	197	4.8
niger No. 2	250	39	6.4
flavus No. 1	400	0.61	660
Yeast (invertase)	86,000	20	4300

It is desirable that additional experiments on the purification of such preparations be carried out so that the identity or nonidentity of invertases and inulases may be settled. Since Weidenhagen (69) was unable to separate them, the writer prefers to consider that they probably are identical but vary according to their origin.

It is of interest that the highly active invertase preparations of Adams, Richtmyer, and Hudson (1), in confirmation of earlier results, exhibit no hydrolytic action on melezitose and on isosucrose. If the melezitose is hydrolyzed by invertase, the rate must be less than one-millionth that of sucrose. The lack of hydrolysis of isosucrose (β-glucopyranosyl-α-fructofuranoside) is to be ascribed to the presence of an alpha- rather than a beta-fructosidic linkage, although it has been recently claimed (60) that isosucrose is a fructose glucoside rather than a fructosyl-glucoside. Other

α-fructofuranosides, *e. g.*, the methyl and benzyl fructosides, and β-fructo-pyranosides are unaffected by yeast invertase preparations (59, 69).

V. Weidenhagen Theory and Classification of the Carbohydrases

1. The Weidenhagen Theory

Weidenhagen has presented a system for the classification of the carbo-hydrases; and, although the system has proved of value and has stimulated some research, it has received considerable criticism, much of which is well founded. In its original form (66, 68), the system demanded that there be only one enzyme for each glycosidic type. One enzyme (β-glucosidase) should hydrolyze all β-D-glucosides, one (α-glucosidase) all α-glucosides, one (α-galactosidase) all α-galactosides, etc. The disaccha-rides are considered as glycosides and the same enzyme was originally made responsible for the hydrolysis of related disaccharides, glycosides, and polysaccharides. The system required that β-glucosidase hydrolyze alkyl and aryl β-D-glucosides, cellobiose, gentiobiose, and cellulose. Similarly, α-glucosidase should catalyze the hydrolysis of alkyl and aryl α-D-gluco-sides and maltose; also invertase should cleave both sucrose and inulin. The theory later was modified slightly (67) so as to admit the possibility of polysaccharidases different from glycosidases.

The main defect of the system is the insistence on the identity of enzymes from different sources but acting on the same substrates. Since Weidenhagen ascribes these to differences, not in the enzyme but in the "carrier," the discussion to a consider-able extent is based on the definition of an enzyme. The present writer takes the view that, since no authentic examples of the dissociation of carbohydrases into enzymes and carriers or proteins and coenzymes have been demonstrated, the entire molecule must be termed "the enzyme." Therefore, the variation in any part of the molecule would create a new molecular type and, hence, a new enzyme. A careful study (20) of the ef-fect of the dialysis of β-glucosidase on its activity makes it improbable that this enzyme has a dissociable "carrier." If this is considered as a proper answer to Weidenhagen's explanation, then there is no question that different enzymes exist which act on the same substrates. As has been illustrated in the earlier sections of this paper, the specificities of β-glucosidases, α- and β-galactosidases, invertases, etc., vary according to the origin of the enzymes. Within a given class, the enzymes also show appreciable differences in the pH value for optimal activity, in their adsorptive properties, etc. The reasons for the differences between the enzymes of the various glycosidic types, *e. g.*, β-glucosidases, are unknown, but it is believed that the results obtained in the study of the cleavage of the alkyl β-glucosides by β-glucosidases (page 54) is of importance in this respect. For these compounds, there is an optimal chain length at which the rate of hydrolysis is the greatest. It seems probable that the different enzymes of each glycosidic type differ primarily in the structure of the aglycon-adsorbing area. The enzymes of a given class

would then correspond to different aglycon structures at the region of maximal rate of hydrolysis. The organism elaborating the enzymes might be expected to synthesize enzymes corresponding to the compounds actually being utilized.

2. Classification of Carbohydrases

In Table XVI, a provisional classification of the carbohydrases is given as suggested (54) by the writer.

The carbohydrases require much more investigation before a completely satisfactory classification can be given. That in Table XVI should be considered as provisional and subject to change as more information becomes available. Its principal value is to summarize the present knowledge of the subject and to stimulate additional investigations.

The classification of the amylases is somewhat different from that given earlier (54). The liquefying enzymes have been divided into classes according to their ability to produce fermentable substances. Although this difference is not sharp, unpublished work by the author and M. G. Blair indicates an apparently fundamental difference between the two groups. The liquefying enzymes of the first group appear to be able to convert starches completely to fermentable substances in the presence of yeasts, while the other group is not able to do so.*

The classification of the pectic enzymes follows that proposed by the Committee on the Nomenclature of Pectin.** In the preceding publication (54) it was erroneously ascribed to Z. I. Kertész.†

The principal differences between the system of Table XVI and those of Weidenhagen are: (1) The individual enzymes of Weidenhagen's system are now considered as classes acting on the same substrates but with different specificities and other properties; and (2) the more recent knowledge of the action of glycosidases on pentoses, hexoses, and heptoses of similar structures and configuration is introduced into the system of classification. For the sake of completeness, a more or less standard classification of the polysaccharidases has also been included.

The classification of the glycosidases is based upon the following postulates, some adequately and some inadequately established:

1. Changes in the aglycon group affect the rate of enzymatic hydrolysis of the glycoside; but profound changes are required before the rate becomes inappreciable (aglycon specificity).

2. Glycosides, having different configurations for the glycosidic carbon, require different enzymes (alpha-beta specificity).

3. Enzymes, which are from different sources and which catalyze the same reactions, vary in the specificity which they exhibit.

---

* The writer wishes to express his appreciation of the numerous suggestions and comments made by Dr. Eric Kneen concerning the classification of the amylases.

** *J. Am. Chem. Soc.*, 49, No. 5, p. 37 of the proceedings (1927).

† *Ergeb. Enzymforsch.*, 5, 233 (1936).

TABLE XVI

PROVISIONAL CLASSIFICATION OF THE CARBOHYDRASES

Enzyme class	Other or older names for members of class	Substrates*
GLYCOSIDASES HYDROLYZING SIMPLE GLYCOSIDES AND OLIGOSACCHARIDES**		
β-Glucosidases.......	Emulsin, cellobiases, gentiobiases, prunase, probably β-glucuronidases	β-D-Glucosides, β-D-xylosides, cellobiose, gentiobiose, β-D-glucuronides (?), heptosides with β-D-glucose configuration (?)
α-Glucosidases........	Maltases, probably trehalases	α-D-Glucosides, α-D-xylosides (?), maltose, probably trehalose and sucrose, heptosides with α-D-glucose configuration (?)
β-Galactosidases......	Lactases.............	β-D-Galactosides, α-L-arabinosides, β-D-fucosides (?), lactose, heptosides with β-D-galactose configuration, β-D-galacturonides (?)
α-Galactosidases......	Melibiases..........	α-D-Galactosides, β-L-arabinosides, α-D-fucosides (?), melibiose, heptosides with α-D-galactose configuration (?)
β-Fructofuranosidases or invertases........	Sucrase, saccharase...	β-Fructofuranosides, sucrose, possibly inulin
α-Mannosidases.......	....	α-D-Mannosides, α-D-lyxosides, heptosides with α-D-mannose configuration (?)
β-Thioglucosidases....	Myrosin.............	β-Thioglucosides, thioxylosides (?)
Nucleosidases........	....	N-Glycosides (?) (particularly D-ribose derivatives), nucleosides
POLYSACCHARIDASES**		
Amylases............ Saccharifying amylases (with but slight liquefying power) Liquefying amylases	Diastases........... β-Amylases of wheat, barley, soybeans, etc. Dextrogenic amylases	Starches, glycogen Unbranched chains of maltose residues
Group 1	Malt and certain other cereal alpha-amylases; Aspergillus amylases	Starch substances and probably glycogen
Group 2	Pancreatic amylase, Bacillus macerans amylase, B. mesentericus amylase, possibly salivary amylase	Starch substances and probably glycogen
Phosphoamylases 1. Phosphorylases of Hanes and Cori	....	Starches and glycogen by processes involving phosphorylation
2. Disaggregating amylase of Waldschmidt-Leitz and Mayer	....	Starches are broken by process involving phosphorylation into large molecules. (Existence is questionable)

TABLE XVI (*Continued*)

PROVISIONAL CLASSIFICATION OF THE CARBOHYDRASES

Enzyme class	Other or older names for members of class	Substrates*
Inulases (possibly identical with invertases)	....	Inulin, and poly-β-fructofuranosides
Cellulases...........	....	Cellulose and probably other poly-β-glucosides
Hexosanases, pentosanases, etc...........	Cytases..............	Hexosans, pentosans, etc.
Chitinases...........	....	Chitin and related substances
Pectic enzymes........	....	Pectic substances
Protopectinases.....	....	Hydrolyze native pectins *in situ* to soluble pectins
Pectinases..........	Pectolase...........	Pectins and polygalacturonides
Pectase............	....	An esterase hydrolyzing ester linkages of pectins and of esterified polygalacturonides

* As far as is known, the substances followed by question marks have not been tested, at least not under sufficiently drastic conditions to indicate "unhydrolyzability." The others have been tested for at least one member of the class, usually the corresponding enzyme of almond emulsin.
The list of substrates is not complete but it is intended to represent the more important types.
** There is probably overlapping in the action of the two main classes of carbohydrases but, in general, the action of the one group of enzymes on the substrates of the second class appears to be small and usually may be neglected.

4. Substitution of the ring hydroxyls of hydrolyzable glycosides by other groups makes the glycosides unhydrolyzable by the same enzyme.

5. A change in the configuration of one or more ring carbons or of the ring type (*e. g.*, from pyranose to furanose) makes a hydrolyzable glycoside unhydrolyzable by the same enzyme.

6. Changes made outside of the sugar ring affect the rate of enzymatic hydrolysis, but usually do not result in requiring a new enzyme for the hydrolysis.

7. In natural products, only those enzymes are to be expected which are capable of hydrolyzing glycosides of the same basic ring types as those of naturally occurring glycosides.

8. *Thio-* and *N*-glycosides, with glycosidic linkages taking place through a sulfur or nitrogen atom rather than an oxygen atom, require enzymes different from those hydrolyzing the true glycosides.

Some of these postulates are corollaries of others. Thus, 2 is a special case of 5 and 1 of 6, but their importance makes it desirable to list them separately. Much of the evidence for these assumptions is given in the preceding sections. For additional material, the original reference should be consulted.

3.   Nomenclature of Carbohydrases

In the preceding discussion, the term "emulsin" has been used to replace the expression "crude or partially purified mixtures of enzymes" or "enzyme preparations." Thus, alfalfa emulsin, a product obtained from alfalfa seeds, contains at least several enzymes as well as other material. Almond emulsin is not an enzyme (β-glucosidase), as it has sometimes been employed, but instead is a mixture of enzymes and other material. In general, this usage follows the suggestion of Helferich (37) who employs it to describe partially purified mixtures of glycosidic-hydrolyzing enzymes obtained from plant or animal extracts. The present writer (53), however, does not limit it to mixtures containing carbohydrases but has used it as a general term for mixtures of enzymes.

The main classes of glycosidic-hydrolyzing enzymes are designated according to the alpha or beta type involved as α- or β-hexosidases. In some instances it may be preferable to use other types, but hexose types should be used when possible. Since the enzymes usually act on the D-pyranosides, these designations are usually omitted unless the L-isomers or the furanose isomers are involved.

The suggestion was originally made by Duclaux (4) that individual enzymes be named by adding the suffix "ase" to the name of the substrate on which they act. This has been done in the naming of the classes of glycosidases, but general application to individual enzymes seems undesirable since the action of none of the known glycosidases is limited to a single substrate; and, until all possible aglycon structures have been studied, it would not be known which is the most easily hydrolyzed substrate.

It is suggested that, until more information concerning individual enzymes is available, the individual glycosidases be named according to their source and general type of action. Some of the common enzymes, according to this system are: sweet-almond β-glucosidase, yeast fructofuranosidase (yeast invertase), yeast α-glucosidase (yeast maltase), yeast α-galactosidase (yeast melibiase), wheat β-amylase, malt α-amylase, *Aspergillus niger* inulase, etc.

Bibliography

1.   Adams, M., Richtmyer, N. K., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1369 (1943).

2.   Bourquelot, E., *J. pharm. chim.*, **25**, 378 (1907).

3.   Burk, R. E., *J. Phys. Chem.*, **30**, 1134 (1926).

4. Duclaux, E., *Microbiologie*, p. 141 (1883); quoted by J. B. S. Haldane (see p. 192 of reference 8).

5. von Euler, H., *Z. physiol. Chem.*, **143**, 79 (1925).

6. Fischer, E., *Ber.*, **27**, 2985 (1894).

7. Fischer, E., *Z. physiol. Chem.*, **26**, 60 (1898).

8. Haldane, J. B. S., *Enzymes*. Longmans, Green, London, 1930, p. 182.

9. Helferich, B., *Ergeb. Enzymforsch.*, **7**, 83 (1938).

10. Helferich, B., Appel, H., and·Gootz, R., *Z. physiol. Chem.*, **215**, 277 (1933); Helferich, B., and Winkler, S., *ibid.*, **209**, 269 (1932).

11. Helferich, B., and Brieger, G., *ibid.*, **221**, 94 (1933).

12. Helferich, B., and Burt, C. P., *Ann.*, **520**, 156 (1935).

13. Helferich, B., and Goerdeler, J., *Ber. Verhandl. sächs. Akad. Wiss. Leipzig Math.-phys. Klasse*, **92**, 75 (1940).

14. Helferich, B., and Griebel, R., *Ann.*, **544**, 191 (1940).

15. Helferich, B., and Grünler, S., *J. prakt. Chem.*, **148**, 107 (1937).

16. Helferich, B., Grünler, S., and Gnüchtel, A., *Z. physiol. Chem.*, **248**, 85 (1937).

17. Helferich, B., Günther, E., and Pigman, W. W., *Ber.*, **72**, 1953 (1939).

18. Helferich, B., Günther, E., and Winkler, S., *Ann.*, **508**, 192 (1933).

19. Helferich, B., and Hiltmann, R., *ibid.*, **531**, 160 (1937).

20. Helferich, B., Hiltmann, R., and Pigman, W. W., *Z. physiol. Chem.*, **259**, 150 (1939).

21. Helferich, B., Heyne, H., and Gootz, R., *ibid.*, **214**, 139 (1933).

22. Helferich, B., and Iloff, A., *ibid.*, **221**, 252 (1933).

23. Helferich, B., Iloff, A., and Streeck, H., *ibid.*, **226**, 258 (1934).

24. Helferich, B., and Lampert, U., *Ber.*, **67**, 1667 (1934); **68**, 1266 (1935).

25. Helferich, B., and Lang, O., *Z. physiol. Chem.*, **216**, 123 (1933).

26. Helferich, B., and Lutzmann, H., *Ann.*, **537**, 11 (1938).

27. Helferich, B., and Lutzmann, H., *ibid.*, **541**, 1 (1939).

28. Helferich, B., and Peters, O., *J. prakt. Chem.*, **138**, 281 (1933).

29. Helferich, B., and Philipp, F., *Ann.*, **514**, 228 (1934).

30. Helferich, B., and Pigman, W. W., *Z. physiol. Chem.*, **259**, 253 (1939).

31. Helferich, B., Pigman, W. W., and Isbell, H. S., *ibid.*, **261**, 55 (1939).

32. Helferich, B., and Reischel, W., *Ann.*, **533**, 278 (1938).

33. Helferich, B., and Scheiber, H., *Z. physiol. Chem.*, **226**, 272 (1934).

34. Helferich, B., Scheiber, H. E., Streeck, R., and Vorsatz, F., *Ann.*, **518**, 211 (1935).

35. Helferich, B., and Schnorr, H., *ibid.*, **547**, 201 (1941).

36. Helferich, B., and Sparmberg, G., *Z. physiol. Chem.*, **221**, 92 (1933).

37. Helferich, B., and Vorsatz, F., *ibid.*, **237**, 254 (1935).

38. Helferich, B., and Winkler, S., *Ber.*, **66**, 1556 (1933).

39. Helferich, B., Winkler, S., Gootz, R., Peters, O., and Günther, E., *Z. physiol. Chem.*, **208**, 91 (1932).

40. Hill, K., *Ber. Verhandl. sächs. Akad. Wiss. Leipzig Math.-phys. Klasse*, **86**, 115 (1934).

41. Hitchcock, D. I., *J. Am. Chem. Soc.*, **48**, 2870 (1926).

42. Hofmann, E., *Biochem. Z.*, **285**, 429 (1936).

43. Isbell, H. S., and Pigman, W. W., *J. Research Natl. Bur. Standards*, **18**, 141 (1937).

44. Isbell, H. S., and Frush, H. L., *ibid.*, **24**, 125 (1940).

45. Josephson, K., *Z. physiol. Chem.*, **147**, 9 (1925).
46. Medwedew, G., *Enzymologia*, **2**, 53 (1937–1938).
47. Miwa, T., Cheng, C., Fujisaki, M., and Toishi, A., *Acta Phytochim. Japan*, **10**, 155 (1937).
48. Moelwyn-Hughes, E. A., *Trans. Faraday Soc.*, **25**, 81 (1929).
49. Petersen, S. R., *Ber. Verhandl. sächs. Akad. Wiss. Leipzig Math.-phys. Klasse*, **85**, 154 (1933).
50. Pigman, W. W., *J. Am. Chem. Soc.*, **62**, 1371 (1940).
51. Pigman, W. W., *J. Research Natl. Bur. Standards*, **26**, 197 (1941).
52. Pigman, W. W., *ibid.*, **27**, 1 (1941).
53. Pigman, W. W., *ibid.*, **30**, 159 (1943).
54. Pigman, W. W., *ibid.*, **30**, 257 (1943).
55. Pigman, W. W., *Z. physiol. Chem.*, **261**, 82 (1939).
56. Pigman, W. W., Isbell, H. S., *J. Research Natl. Bur. Standards*, **27**, 9 (1941).
57. Pigman, W. W., and Richtmyer, N. K., *J. Am. Chem. Soc.*, **64**, 369 (1942).
58. Pigman, W. W., and Richtmyer, N. K., *ibid.*, **64**, 374 (1942).
59. Purves, C., and Hudson, C. S., *ibid.*, **56**, 708 (1934); **59**, 49 (1937).
60. Schlubach, H., and Middelhoff, B., *Ann.*, **550**, 134 (1942).
61. Stearn, A. E., *Ergeb. Enzymforsch.*, **7**, 1 (1938).
62. Tauber, H., *J. Biol. Chem.*, **99**, 257 (1932–1933).
63. Veibel, S., and Frederiksen, E., *Kgl. Danske Videnskab. Selskab. Math.-fys. Medd.*, **19** (1) (1941).
64. Veibel, S., and Lillelund, H., *ibid.*, **17** (6) (1940).
65. Veibel, S., and Lillelund, H., *Z. physiol. Chem.*, **253**, 55 (1938).
66. Weidenhagen, R., *Ergeb. Enzymforsch.*, **1**, 168 (1932).
67. Weidenhagen, R., in Nord-Weidenhagen, *Handbuch der Enzymologie*. Akadem. Verlagsgesellschaft, Leipzig, 1940, p. 519.
68. Weidenhagen, R., *Z. Ver. deut. Zucker-Ind.*, **82**, 316 (1932).
69. Weidenhagen, R., *ibid.*, **82**, 912 (1932).
70. Weidenhagen, R., and Renner, A., *ibid.*, **86**, 22 (1936).

THE TRANSAMINATION REACTION

By

ROBERT M. HERBST

Short Hills, New Jersey

CONTENTS

I.   Introduction

The intermolecular transfer of an amino group from an α-amino acid to an α-keto acid has become known as the transamination reaction.   The reaction appears to be a special case of the oxidative deamination of α-amino acids brought about by a variety of organic carbonyl compounds of such diversity as alloxan (63), isatin (29, 55, 64), quinones (47, 64), ninhydrin (1, 16, 32, 33, 60, 61), and α-dicarbonyls such as methylglyoxal and phenylglyoxal (59).   The α-amino nitrogen is liberated as ammonia in all of these systems except in the case of ninhydrin, where the ammonia

75

formed in the primary reaction undergoes further reaction with the reagent.

A typical transamination was first observed by Herbst and Engel (38) in experiments with model systems in which the more reactive α-dicarbonyl derivatives were replaced by an α-keto acid. Here the amino acid again suffered oxidative deamination, but the nitrogen reappeared in combination with the keto acid which had undergone reductive amination. Soon thereafter Braunshtein and Kritsman (9) observed that a similar reaction was catalyzed by the enzymes of certain tissues, and suggested the name "Umaminierung"—transamination—for the reaction.

These results had been foreshadowed by occasional observations pointing toward the existence of unknown metabolic conversions of amino acids. Observations such as those of Needham (58) and Holmberg (41) indicated that various tissues could bring about the oxidation of glutamic acid without the formation of detectable amounts of ammonia. For instance, Needham showed that glutamic and aspartic acids were converted into succinic acid by muscle tissue but that the total amino nitrogen content of the system remained constant. She suggested that the amino nitrogen of the dicarboxylic acids was taken up by "some reactive carbohydrate residue" without the intermediate formation of ammonia.

Transamination in model systems is differentiated sharply from enzyme-catalyzed transamination by the fact that the amino acid suffers, in the former case, not only oxidative deamination but decarboxylation as well, while in the latter case no carbon dioxide is formed. This indicates a rather profound difference between the mechanisms of the two processes and provides a natural subdivision for the following discussion.

II. Transamination in Model Systems

When a mixture of α-aminophenylacetic acid and pyruvic acid in water in a test tube is boiled for a few minutes, the odor of benzaldehyde betrays the occurrence of a reaction. A more quantitative investigation (38) of this simple observation shows that approximately equimolar quantities of benzaldehyde, carbon dioxide, and alanine are formed. The over-all effect of the reaction may be expressed as follows:

$$C_6H_5CH(NH_2)COOH + CH_3COCOOH \longrightarrow$$
$$C_6H_5CHO + CO_2 + CH_3CH(NH_2)COOH$$
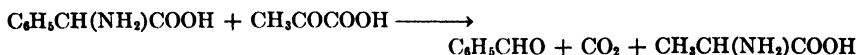
Addition of mineral acids to the reaction mixture caused almost no inhibition, whereas addition of alkali in sufficient quantity to convert the organic acids into their salts effected complete inhibition of the reaction.

The results of studies of the reaction between a number of keto acid–amino acid combinations (35, 38) are summarized in Table I. It should be pointed out that the reaction always led to the formation of racemic products. Even when optically active amino acids were used, the newly

TABLE I

REACTIONS BETWEEN COMBINATIONS OF KETO AND AMINO ACIDS (37)

Reactants	Products
Pyruvic acid plus:	
Glycine	Formaldehyde, glyoxylic acid, carbon dioxide, alanine.
Alanine	Acetaldehyde, carbon dioxide.
Leucine	Isovaleraldehyde, carbon dioxide, alanine.
α-Aminophenylacetic acid	Benzaldehyde, carbon dioxide, alanine.
α-Amino-*p*-methoxyphenylacetic acid	Anisaldehyde, acetaldehyde, carbon dioxide, alanine.
Phenylalanine	Phenylacetaldehyde, carbon dioxide, alanine.
p-Methoxyphenylalanine	*p*-Methoxyphenylacetaldehyde, acetaldehyde, carbon dioxide, alanine.
Aspartic acid	Acetaldehyde, carbon dioxide, alanine.
Glutamic acid	Carbon dioxide, alanine.
Cystine	Acetaldehyde, carbon dioxide, alanine.
S-Ethylcysteine	Ethylthioglycolaldehyde, acetaldehyde, carbon dioxide, alanine.
S-Phenylcysteine	Phenylthioglycolaldehyde, acetaldehyde, carbon dioxide, alanine.
S-Benzylcysteine	Benzylthioglycolaldehyde, acetaldehyde, carbon dioxide, alanine.
α-Amino-isobutyric acid	Acetaldehyde, carbon dioxide (traces).
α-Amino-α-phenylbutyric acid	Propiophenone, acetaldehyde, carbon dioxide, alanine.
α-Methylaminophenylacetic acid	No reaction.
Phenylpyruvic acid plus:	
α-Aminophenylacetic acid	Benzaldehyde, carbon dioxide, phenylalanine.
Cystine	Carbon dioxide, phenylalanine.
zoylformic acid plus:	
Alanine	Practically no reaction.
α-Amino-*p*-methoxyphenylacetic acid	Benzaldehyde–anisaldehyde mixture, mixed amino acids.
Cystine	Carbon dioxide, α-aminophenylacetic acid.
S-Ethylcysteine	Ethylthioglycolaldehyde.

formed amino acid failed to exhibit optical activity. In fact, the rates of carbon dioxide evolution and the disappearance of optical activity were comparable.

The relative rates of reaction between a number of amino acids and pyruvic acid in boiling aqueous solution, as indicated by the speed of carbon dioxide evolution, are shown in Figures 1 and 2. It can be seen

clearly from the curves in the figures that the amino acids fall into distinct groups with respect to the ease with which they react with pyruvic acid. Rapid reaction is noted in the case of *dl*-α-aminophenylacetic acid and *l*(−)-cystine and its derivatives. The same characteristically rapid reaction with pyruvic acid is exhibited by other ring substituted α-amino-phenylacetic acids (34). *dl*-Phenylalanine and *l*(+)-glutamic acid react only slowly with pyruvic acid, while *l*(−)-aspartic acid assumes an inter-
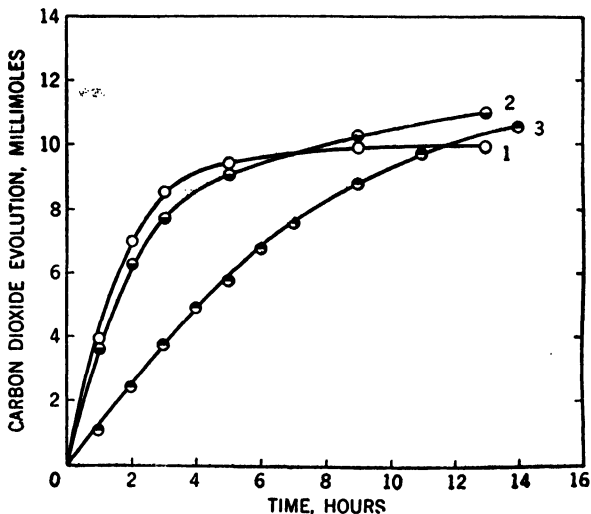
Fig. 1 —Rate of carbon dioxide evolution during the reaction of pyruvic acid (30 mM.) in boiling aqueous solution with: curve 1, *dl*-α-aminophenylacetic acid (10 mM.); curve 2, *l*(−)-cystine (5 mM.); curve 3, *l*(−)-*S*-ethyl-cysteine (10 mM.).

mediate position. The ease with which certain monoamino monocarboxylic acids react with pyruvic acid definitely distinguishes the model reaction from the enzyme-catalyzed reaction.

Interesting also is the difference in the speed of reaction between pyruvic acid and *l*(−)-aspartic acid and *l*(+)-glutamic acid. A partial explanation of the slowness of the reaction with glutamic acid was found in the observation (36) that, under the acid conditions of the reaction, about *p*H 3, glutamic acid was converted quite rapidly into an optically inactive mixture of about two parts of *l*(−)-pyrrolidone carboxylic acid and one part of *l*(+)-glutamic acid. Boiling this mixture after strongly acidifying with hydrochloric acid restored most of the initial optical activity.

The results recorded in Table I indicate certain facts which any mechanism proposed for the reaction must explain. A primary amino group appears to be a prerequisite, since α-methylaminophenylacetic acid failed to react with pyruvic acid. The hydrogen in the alpha position of the original amino acid does not appear to enter into the reaction as indicated by the formation of propiophenone, carbon dioxide, and alanine in the reaction between pyruvic acid and α-amino-α-phenylbutyric acid. Undissociated carboxyl groups appear to favor the reaction, as indicated by
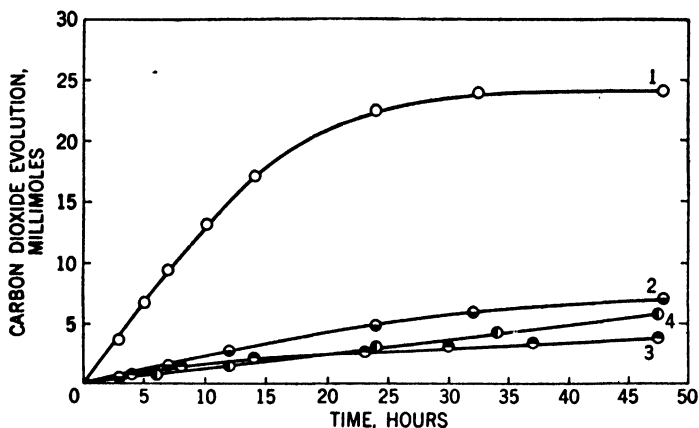
Fig. 2.—Rate of carbon dioxide evolution during the reaction of pyruvic acid (40 mM.) in boiling aqueous solution with: curve 1, $l(-)$-aspartic acid (10 mM.); curve 2, dl-phenylalanine (10 mM.), curve 3, $l(+)$-glutamic acid (10 mM.); curve 4, dl-α-amino-α-phenylbutyric acid (10 mM.).

the inhibiting effect of alkali as contrasted with the near absence of inhibition by mineral acids. Finally, the mechanism must account for the formation of two aldehydes in many examples. The mechanism outlined in Scheme 1 has been suggested (35, 37) for model transaminating systems.

The postulated intermediate of the Schiff's base type may decarboxylate in either of the two ways represented by (A) and (B) in Scheme 1. In case the reaction follows course (A), decarboxylation and the shift of the double bond are considered to take place simultaneously. The nature of the end products is determined, of course, by the relative rates of decarboxylation according to (A) or (B), and this is naturally dependent upon the effect of the substituents R and R' in directing the course of the reaction.

The postulation of an intermediate of the Schiff's base type receives support in the synthesis by Knoop and Martius (48) of an iminodicarboxylic acid of the octopine type by the catalytic hydrogenation of a mixture of $l(+)$-arginine and pyruvic acid in aqueous solution in the presence of palladium. Attempts to repeat this preparation in the author's laboratory have led to the almost exclusive formation of the diastereoisomer, iso-octopine, and no isolatable quantity of octopine (36). Although this in no way vitiates the value of the evidence of Knoop and Martius in support of a Schiff's base-like intermediate, their failure to recognize their product as iso-octopine undoubtedly traces to an erroneous melting point reported for this compound by Akasi (4).

$$
\begin{array}{ccc}
\text{R—CH—NH}_2 & \text{O=C—R'} & \text{R—CH—N=C—R'} \\
\quad\big| & \quad\big| & \quad\big|\qquad\big| \\
\text{COOH} & + \;\; \text{COOH} \;\;\longrightarrow & \text{COOH} \;\; \text{COOH} \;\; + \;\text{H}_2\text{O}
\end{array}
$$

(A) $-CO_2$ (B) $-CO_2$

$$
\begin{array}{cc}
\text{R—CH=N—CH—R'} & \text{R—CH—N=CH—R'} \\
\qquad\big| & \qquad\big| \\
\text{COOH} & \text{COOH}
\end{array}
$$

$+\text{H}_2\text{O} \downarrow \qquad\qquad \downarrow +\text{H}_2\text{O}$

$$
\begin{array}{cc}
\text{R—CHO} + \text{H}_2\text{N—CH—R'} & \text{R—CH—NH}_2 + \text{R'—CHO} \\
\qquad\qquad\big| & \big| \\
\qquad\qquad\text{COOH} & \text{COOH}
\end{array}
$$
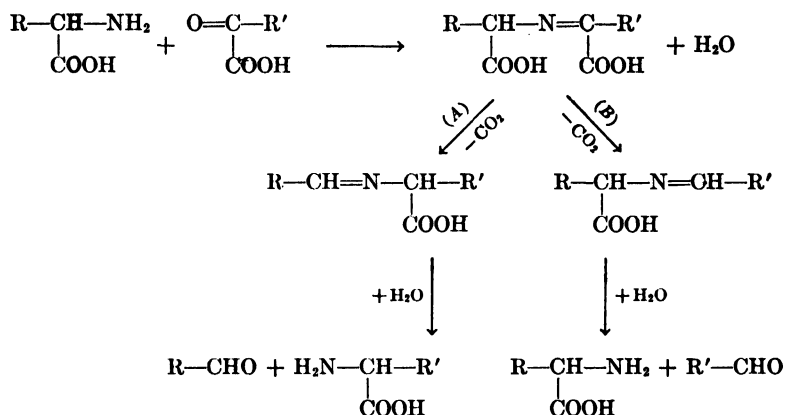
SCHEME 1

The mechanism of the model transamination reaction was recently studied by Herbst and Rittenberg (39) in order to clarify the role of the hydrogen in the alpha position of the original amino acid. When the reaction between α-aminophenylacetic acid and pyruvic acid was carried out in water containing known amounts of deuterium, the alanine formed during the reaction contained deuterium not only in the alpha position but in the beta position as well. The benzaldehyde resulting from the reaction was always free of excess deuterium. These results were checked by a study of the reaction between α-deuterio-α-aminophenylacetic acid and pyruvic acid in ordinary water, when the benzaldehyde contained most of the deuterium present in the original amino acid, while the newly formed alanine was free of excess isotope. The suggestion was again made that decarboxylation and the shift of the double bond were intimately

associated processes and probably occurred simultaneously. The previously proposed mechanism was modified to the extent of suggesting an electromeric shift as indicated in Scheme 2 to account for the shift of the double bond without dissociation of a proton from the alpha carbon atom.

$$R\text{—}CH\text{—}N\text{=}C\text{—}R' \longrightarrow H^+ + CO_2 + \quad R\text{—}CH\text{=}N\text{—}C\text{—}R'$$
$$\underset{COOH}{\mid} \quad \underset{COOH}{\mid} \qquad\qquad\qquad\qquad \underset{COOH}{\mid}$$

$$\xrightarrow{+H^+} \quad R\text{—}CH\text{=}N\text{—}CH\text{—}R' \quad \xrightarrow{+H_2O} \quad R\text{—}CHO + \quad H_2N\text{—}CH\text{—}R'$$
$$\underset{COOH}{\mid} \qquad\qquad\qquad\qquad\qquad \underset{COOH}{\mid}$$
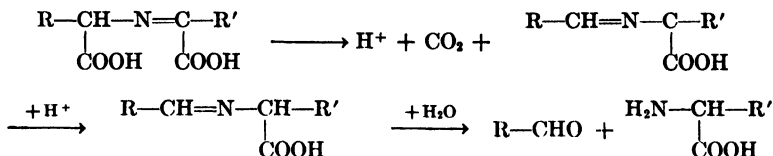
<center>SCHEME 2</center>

The appearance of deuterium in the beta position of the alanine lent further support to the postulation of a Schiff's base as the first step in the reaction. The only apparent explanation for this observation involved tautomerism of such an intermediate, thus bringing the beta hydrogens into a "labile" position as indicated in Scheme 3.

$$R\text{—}CH\text{—}N\text{=}C\text{—}CH_3 \quad \underset{\longleftarrow}{\longrightarrow} \quad R\text{—}CH\text{—}NH\text{—}C\text{=}CH_2$$
$$\underset{COOH}{\mid} \quad \underset{COOH}{\mid} \qquad\qquad\qquad \underset{COOH}{\mid} \quad \underset{COOH}{\mid}$$
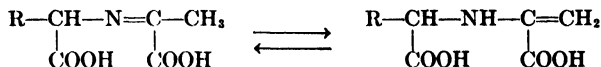
<center>SCHEME 3</center>

Transamination with the esters of amino acids and keto acids was studied by Brewer and Herbst (13) in the hope of finding a model system more closely analogous to the enzyme-catalyzed systems described below. When the carboxyl groups of both the amino acid and the keto acid were blocked by esterification, thus preventing decarboxylation, a tautomeric shift of the alpha hydrogen from the amino ester residue to the keto ester residue was induced in anhydrous media by alkaline catalysts such as sodium ethoxide. Tautomeric shift did not take place in the presence of anhydrous hydrogen chloride. The mechanism outlined in Scheme 4

$$C_6H_5CH(NH_2)COOC_2H_5 \qquad\qquad C_6H_5COCOOC_2H_5$$
$$+ \qquad\qquad\qquad\qquad +$$
$$CH_3COCOOC_2H_5 \qquad\qquad CH_3CH(NH_2)COOC_2H_5$$
$$-H_2O \updownarrow \qquad\qquad -H_2O \updownarrow$$
$$C_6H_5CHCOOC_2H_5 \qquad\qquad C_6H_5CCOOC_2H_5$$
$$\underset{N}{\mid} \qquad\qquad\qquad \underset{N}{\parallel}$$
$$\underset{\parallel}{N} \qquad\qquad$$
$$CH_3CCOOC_2H_5 \qquad\qquad CH_3CHCOOC_2H_5$$

$+H_2O$ HCl $+H_2O$ HCl
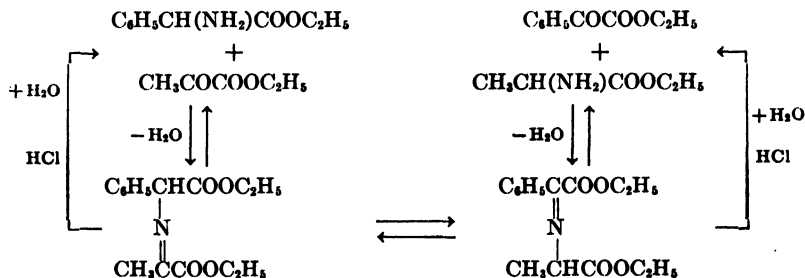
<center>SCHEME 4</center>

has been suggested for this reaction; and it should be remarked that it bears a close relationship to the prototropic methyleneazomethine ($=$CH—N$=$C$<$) systems studied by Ingold and coworkers (42).

The close analogy between the mechanism of the model negative ion-catalyzed transamination (Scheme 4) and that of enzyme-catalyzed transamination (Scheme 6) should be emphasized at this point.

The possibility of peptide synthesis by transamination in model systems has been demonstrated by Herbst and Shemin (40). Amino groups were introduced successively into pyruvic acid and pyruvylalanine by interaction with α-aminophenylacetic acid with the formation of dl-alanine and alanylalanine, respectively, as indicated in Scheme 5. The suggestion was made that aminations of this type might be of importance in the biological synthesis of peptides and proteins.

$$\text{RCOCOOH} \xrightarrow{\text{Transamination}} \text{RCH(NH}_2\text{)COOH} \xrightarrow{\text{R'COCOOH}}$$

$$\begin{array}{c} \text{R'COCONH} \\ | \\ \text{RCHCOOH} \end{array} \xrightarrow{\text{Transamination}} \begin{array}{c} \text{R'CH(NH}_2\text{)CONH} \\ | \\ \text{RCHCOOH} \end{array}$$
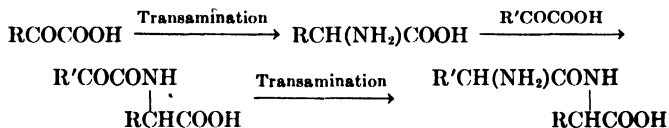
SCHEME 5

III. Enzyme-Catalyzed Transaminations

1. Transamination in Tissue Preparations

In 1937, Braunshtein and Kritsman (9) recognized the presence of an enzyme in muscle tissue which catalyzed the deamination of $l(+)$-glutamic acid under anaerobic conditions with the transfer of the amino group to pyruvic acid and the formation of $l(+)$-alanine. Chopped rabbit and pigeon muscle tissue was used in these early experiments. Under aerobic conditions, the α-ketoglutaric acid formed by the oxidative deamination of glutamic acid underwent further oxidation to succinic acid, while some pyruvic acid was reduced to lactic acid in a coupled oxidation-reduction reaction. Evidence that $l(+)$-alanine was formed by the reaction was adduced from an experiment in which five grams of glutamic acid was incubated with a total of 300 grams of rabbit muscle hash. From this mixture, 200 milligrams of α-naphthalenesulfonylalanine could be isolated. The conclusion that it was optically active was based entirely upon its melting point and the fact that it crystallized with water of hydration.

The reversibility of the reaction was also established by Braunshtein and Kritsman (9), who showed that the same tissues would catalyze a reaction between α-ketoglutaric acid and $l(+)$-alanine with the formation

of pyruvic acid and $l(+)$-glutamic acid. Determination of the optical activity of the product again was made by observation of the melting point of a small quantity of the hydrochloride isolated from a mixture containing 50 grams of rabbit muscle hash, 1.78 grams of dl-alanine, and 1.5 grams of α-ketoglutaric acid.

The enzyme system was not inhibited by $M/100$ sodium arsenite or by bromoacetate in 1:5000 concentration.

2. Mechanism of the Reaction

On the basis of their experiments, Braunshtein and Kritsman (9) outlined the mechanism for the reaction shown in Scheme 6.
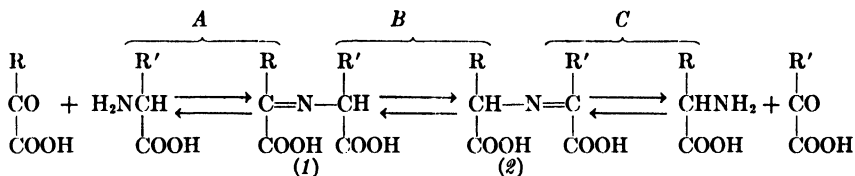
SCHEME 6

The process may be divided into three stages: A, the reversible spontaneous condensation of the components with the formation of a labile Schiff's base (1); B, the reversible rearrangement of (1) and (2) which may be considered as an intramolecular oxidation-reduction catalyzed by the enzyme system; and C, the reversible hydrolysis of (2) into its components, which may be considered the counterpart of A.

Evidence for the correctness of the mechanism outlined in Scheme 6 has recently been presented by Konikova, Kritsman, and Teis (50), who studied the reaction between α-deuterioalanine and α-ketoglutaric acid with a purified enzyme system. Most of the deuterium passed into the aqueous reaction medium during the reaction, while the newly formed glutamic acid contained almost no excess of deuterium. In control experiments without enzyme or without α-ketoglutaric acid, there was no appreciable loss of isotope from the α-deuterioalanine. These results were to be anticipated if hydrogen migrated across the methyleneazomethine bridge, since such a mechanism would require the primary dissociation of a proton from the alpha carbon atom. It is probable that the enzyme-catalyzed interconversion of the two Schiff's bases, (1) and (2), proceeds according to the mechanism outlined in Scheme 7.

$$
\begin{array}{ccc}
\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{C}}}}=\text{N}-\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{CH}}}} & \rightleftharpoons & \text{H}^+ + \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{C}}}}=\text{N}-\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{C}^-}}} & \rightleftharpoons
\end{array}
$$

$$
\begin{array}{ccc}
-\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{C}}}}-\text{N}=\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{C}}}} + \text{H}^+ & \rightleftharpoons & \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{CH}}}}-\text{N}=\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{C}}}}
\end{array}
$$

(1)   (2)
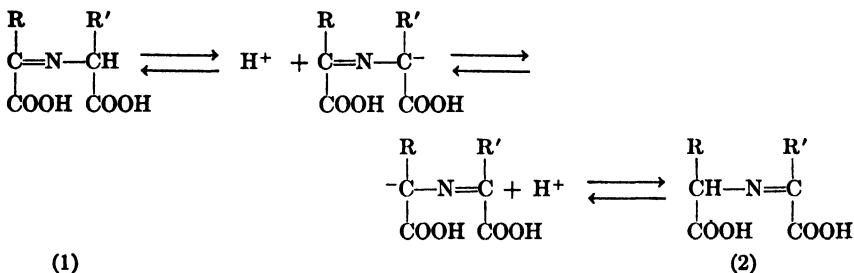
SCHEME 7

From this scheme it appears that the mechanism of the enzyme-catalyzed transaminations is closely analogous to that of the negative ion-catalyzed transamination observed in the case of ethyl pyruvate and the ethyl ester of α-aminophenylacetic acid (13).

On the basis of an observation that fresh liver hash contained an *l*-amino acid dehydrogenase capable of dehydrogenating octopine, a naturally occurring α,α'-iminodicarboxylic acid carrying the carbon chains of *l*(+)-arginine and alanine attached to the imino nitrogen, Karrer and coworkers (44) postulated the mechanism outlined in Scheme 8 for transaminating systems.

$$
\begin{array}{ccc}
\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{CO}}}} + \text{H}_2\text{N}\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{CH}}}} & \rightleftharpoons & \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{C}}}}=\text{N}-\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{CH}}}} & \underset{-2\text{H}}{\overset{+2\text{H}}{\rightleftharpoons}} & \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{CH}}}}-\text{NH}-\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{CH}}}}
\end{array}
$$

$$
\begin{array}{ccc}
\underset{+2\text{H}}{\overset{-2\text{H}}{\rightleftharpoons}} & \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{CH}}}}-\text{N}=\underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{C}}}} & \rightleftharpoons & \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}}{\underset{|}{\text{CHNH}_2}}} + \underset{\substack{|\\ \text{COOH}}}{\overset{\text{R}'}{\underset{|}{\text{CO}}}}
\end{array}
$$
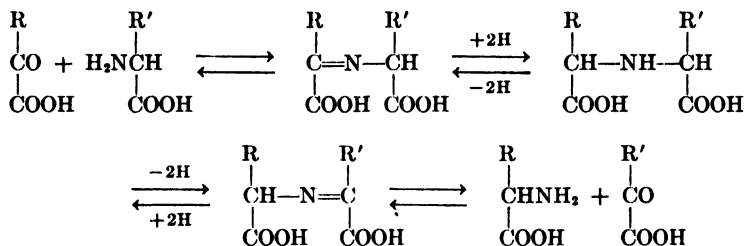
SCHEME 8

The scheme involves hydrogenation of the Schiff's base to an iminodicarboxylic acid, followed by dehydrogenation in either of two positions and hydrolysis of the newly formed Schiff's base. The suggestion was based on the hope that the transaminating enzymes might be *l*-amino acid dehydrogenases. Although experiments with the action of extracts of hog and sheep kidneys on α,α'-iminodipropionic acid (45) seemed to confirm this thought, subsequent more careful experiments with the same materials (43) failed to agree with the earlier results, and the suggestion was retracted.

3. Scope of the Reaction

In their early publications, Braunshtein and Kritsman maintained that enzymatic transamination took place between almost any α-amino acid and α-ketoglutaric acid (9), with the possible exception of glycine (10). Likewise, a variety of α-keto acids were reported capable of accepting the amino groups of glutamic and aspartic acids. On the other hand, amines and peptides (10) could not transfer amino groups to α-keto acids, nor could various ketones, hydroxy ketones, or aldehydes act as amino group acceptors. Later, Karyagina (46), working in Braunshtein's laboratory, presented evidence to the effect that $l(-)$-aspartic acid would undergo intermolecular amino group transfer with pyruvic acid. Karyagina suggested that at least two enyzme systems were active in catalyzing transamination reactions, one of which was specifically active in catalyzing reactions involving glutamic acid or α-ketoglutaric acid, the other effecting amino group transfers involving aspartic acid or oxalacetic acid. It had become clear by then that *one member* of a transaminating pair must be either a dicarboxylic keto or amino acid, since no amino group transfer had been observed between monocarboxylic keto and amino acids.

Braunshtein and Kritsman (11) have reported the interesting observation that, although amino group transfer does not take place between monocarboxylic keto and amino acids, the addition of a dicarboxylic amino or keto acid, specifically $l(+)$-glutamic acid or α-ketoglutaric acid, will catalyze transamination between such a pair.

Cohen (19) failed to find the transamination reaction to be as general as indicated by the work of Braunshtein and his collaborators. For instance, using pigeon breast muscle as the source of the enzyme systems, he reported that of 21 amino acids studied, only $l(-)$-aspartic acid and $l(+)$-alanine readily transferred their amino groups to α-ketoglutaric acid, while dl-α-aminobutyric acid and $l(+)$-valine appeared to be slightly active. None of the other 17 amino acids tested showed appreciable activity. Only oxalacetic acid and pyruvic acid showed pronounced activity as amino group acceptors with glutamic acid. α-Ketoglutaric acid was, of course, active; α-ketobutyric acid and mesoxalic acid showed slight activity; while α-ketovaleric, α-keto-hexanoic, acetoacetic, and levulinic acids showed no appreciable acceptor activity.

4. Analytical Methods

Of necessity, much of the work on enzymatic transamination has been of a qualitative nature because of the difficulty of quantitatively estimating small amounts of amino acids. Specific quantitative analytical methods are available for relatively few of the amino acids, and unfortunately few of these are prominently concerned with the transamination reaction.

Most of the confusion and the contradictory results of various authors can be traced directly to this cause.

Until recently, no specific quantitative method was available for the estimation of glutamic acid. Braunshtein and his coworkers have employed the change in the amino nitrogen content (Van Slyke) of a partially purified, alcohol-insoluble barium salt precipitate (Forman fraction) of the dicarboxylic acids. Recently they used (7) a similar calcium salt fraction, after showing that the precipitate of calcium salts carries down a considerably lesser amount of monoamino monocarboxylic acids than the barium salt precipitate, especially in the presence of larger amounts of the simple amino acids.

Cohen (19) and Zorn (68) have both criticized the analytical methods employed in Braunshtein's laboratory. Cohen (18) has developed an apparently reliable, specific micromethod for the quantitative estimation of glutamic acid. The method involves oxidation of glutamic acid with chloramine-T to β-cyanopropionic acid, hydrolysis of the latter to succinic acid, and estimation of the succinic acid by means of succinoxidase. The final result depends upon the measurement of the disappearance of a few microliters of oxygen and the formation of a few microliters of carbon dioxide. A more specific method for the estimation of small amounts of aspartic acid has also been described by Cohen (20).

5. Isolation of Enzyme Systems

The name "aminopherase" has been applied by Braunshtein and coworkers to the enzyme systems catalyzing amino group transfer. A partially purified enzyme preparation which will catalyze amino group transfers involving $l(+)$-glutamic acid or α-ketoglutaric acid has been described by Kritsman (51). The preparation may be stored as a dry powder for periods of a month, at which time it still retains 80–85% of its original activity. Maximum activity is exhibited at pH 7.5. Equally active preparations have been obtained by extraction of pigeon breast muscle and from pig heart, while rabbit muscle yielded a slightly less active preparation. Because of the specificity of this enzyme preparation toward amino group transfers involving glutamic acid or α-ketoglutaric acid, it has been called glutamic aminopherase.

A second enzyme preparation, described by Kritsman (53, 54) as aspartic aminopherase, was obtained from pig heart, or better by extraction of *coarsely ground* pea seedlings. The preparation from pig heart is usually accompanied by glutamic aminopherase, as is the preparation obtained by extraction of *finely ground* pea seedlings. Its activity is directed specifically toward the catalysis of transaminations involving $l(-)$-aspartic acid and oxalacetic acid.

Both enzyme systems are thermolabile and are easily inactivated by adsorption, dialysis, or salting out. Reactivation, except in the case of

thermal inactivation, is said to be induced (54) by the addition of a thermostabile activator or coenzyme of low molecular weight present in boiled muscle extracts or ultrafiltrates.

The effect of a variety of specific poisons and chemical agents upon the activity of glutamic aminopherase has been determined by Vyshepan (66).

Cohen (20) has described an enzyme preparation obtained from both pigeon breast muscle and pig heart muscle for which he proposed the more euphonious name "transaminase." The preparation appears to be similar to the glutamic aminopherase of Kritsman, and shows some of the properties of aspartic aminopherase as well. Cohen is of the opinion that only one transaminase exists, and that the apparent existence of two enzymes is a difference in degree of activity rather than in specificity.

6. Specificity of Enzyme Systems

Although the various enzyme systems are active primarily in catalyzing the transfer of amino groups where l-amino acids are involved, they have been reported to show slight activity toward amino acids of the d-configurational series (5, 6, 28). However, Cohen (19, 20), using more specific analytical methods, was not able to find evidence of appreciable amounts of transamination with d-amino acids when using either minced pigeon breast muscle or purified enzyme preparations. It is very probable that the earlier contrary findings are due to inaccuracies brought about by the analytical methods employed.

Bychov (14) has reported that certain derivatives of monoamino monocarboxylic acids, such as $l(-)$-cysteic acid and phosphoserine, can replace glutamic and aspartic acids as amino group donors. However, these observations are limited to experiments with minced tissue, since the purified enzyme preparations failed to catalyze the reactions. Cohen (20) has confirmed these findings in regard to cysteic acid with transaminase solutions, but failed to find any evidence of transamination with phosphoserine and either α-ketoglutaric acid or oxalacetic acid under the same conditions.

The kinetics of transaminase activity in extracts of a number of tissues have been studied by Cohen (21) and Cohen and Hekhuis (24). They concluded that transamination was limited largely to the three reactions indicated in the accompanying outline. Of these, the reaction between $l(+)$-glutamic acid and oxalacetic acid is the most rapid and appears to be quantitatively the most important.

$l(+)$-Glutamic acid + oxalacetic acid   (1)

$$\xrightarrow[b]{a} \text{α-ketoglutaric acid} + l(-)\text{-aspartic acid}$$

$l(+)$-Glutamic acid + pyruvic acid   (2)

$$\xrightarrow[b]{a} \text{α-ketoglutaric acid} + l(+)\text{-alanine}$$

$l(-)$-Aspartic acid + pyruvic acid   (3)

$$\xrightarrow[b]{a} \text{oxalacetic acid} + l(+)\text{-alanine}$$
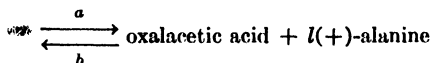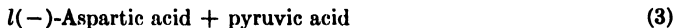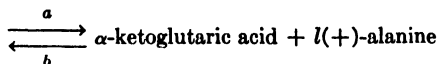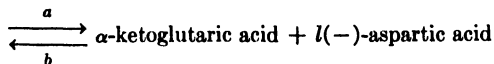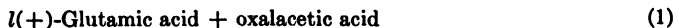
Using his transaminase preparations, Cohen found reaction (1) to be about five times as rapid as reaction (2), while reaction (3) was not catalyzed to an appreciable extent. Reaction (1) alone proceeded at a sufficiently rapid rate to compete successfully with other tissue enzyme systems involved in the intermediary metabolism of amino acids and carbohydrates and acting upon the same substrates. It must be borne in mind that those substrates which appear to be most actively transaminated hold a central position in carbohydrate and amino acid metabolism, and consequently form the substrates for numerous highly active enzyme systems.

7. Distribution of the Enzyme System

Cohen (22) has assembled the data on the transaminating activity of various tissues available in the literature, and has calculated the rates of reactions (1), (2), and (3) on a comparable basis. These data are presented in Table II. The speed of the three reactions is expressed by the transamination quotient, $Q_{transamination}$:

$$Q_{transamination} = \frac{\text{microliters substrate transaminated}}{\text{mg. dry weight of tissue} \times \text{hours}}$$

It can be seen from the data in Table II that the transamination reaction is not limited to animal tissue, but occurs as well in vegetable tissue and in microorganisms of various types. Particularly noteworthy is the very low rate of transamination in tumor tissue as compared with normal tissue. The extremely low rate of amino group transfer in seeds and seedlings would seem to relegate transamination to a relatively unimportant role in these materials. However, the method of extracting and purifying the enzyme preparations may have caused considerable inactivation.

TABLE II

TRANSAMINATION FOR DIFFERENT ANIMAL AND PLANT TISSUES (22)

Type of tissue or microorganism	Reaction No.	$Q_{trans.}$	Reference
1. Animal			
Muscle:			
Skeletal (rat)	1a	316	24
	1b	135	24
	2a	13	24
	2b	4	24
	3a	1	24
	3b	2	24
Skeletal (cat)	1a	20	12
Diaphragm (beef)	2a	3	3
	2b	3	3
Breast (pigeon)	1a	450	23
	1b	160	23
	2a	50	19
	2a	28	9
	2b	42	19
	2b	36	9
	3b	1	5
Heart (rat)	1a	425	24
	1b	190	24
	2a	7	24
	2b	4	24
	3a	7	24
	3b	2	24
Heart (rabbit)	1a	1	52
Heart (dog)	3b	1	5
Liver (rat)	1a	245	24
	1b	102	24
	2a	46	24
	2b	3	24
	3a	10	24
	3b	3	24
Liver (cat)	1a	230	25
	1a	14	12
Liver (rabbit)	2a	1	52
	3b	1	5
Kidney (rat)	1a	245	24
	1b	112	24
	2a	3	24
	2b	3	24
	3a	3	24
	3b	2	24
Kidney (cat)	1a	220	25
	1a	23	12
Kidney (rabbit)	2a	1	52
	3b	1	5
Brain (rat)	1a	260	24
	1b	107	24
	2a	2	24
	2b	3	24
	3a	8	24
	3b	1	24

TABLE II (*Continued*)

TRANSAMINATION FOR DIFFERENT ANIMAL AND PLANT TISSUES (22)

Type of tissue or microorganism	Reaction No.	$Q_{trans.}$	Reference
Brain (cat)	1a	220	25
	1a	20	12
Brain (rabbit)	2a	2	52
	3b	0.5	5
Lung (rat)	1a	51	24
Lung (cat)	1a	23	12
Testis (rat)	1a	150	24
Spleen (rat)	1a	16	24
Erythrocytes (cat)	1a	3	12
Defibrinated blood (human)	1a	0	23
Serum (human)	1a	0	23
Mammary gland, lactating (guinea pig)	2b	3	23
Embryonic (cat):			
Liver	1a	64	25
Kidney	1a	78	25
Brain	1a	77	25
Muscle	1a	20	12
Placenta	1a	94	25
Tumors:			
No. 108 (mouse)	1a	72	25
	1b	3	25
	2a	0.5	25
	2b	0.6	25
Yale No. 1 (mouse)	1a	54	25
	1b	2	25
	2a	0.7	25
	2b	0.6	25
No. 15091-A (mouse)	1a	54	25
	1b	3	25
	2a	0.3	25
	2b	1.0	25
S-37 (mouse)	1a	57	25
	2a	0.7	25
No. 42 (mouse)	1a	72	25
	2a	0.7	25
U.S.P.H.S. No. 17 (mouse)	1a	53	25
	2a	0.5	25
Hepatoma (rat)	1a	64	26
Brown-Pearce carcinoma (rabbit)	2a	0–0.6	6
	2b	0.9	6
Jensen sarcoma (rat)	2a	0–0.8	6
	2b	0.2	6
Sinelnikov-Krichensky sarcoma (rat)	2a	0.4	6
Rous sarcoma (chicken)	2a	0–0.2	6
Fischer sarcoma (chicken)	2a	1	6
	2b	0.2	6

TABLE II (*Continued*)

TRANSAMINATION FOR DIFFERENT ANIMAL AND PLANT TISSUES (22)

Type of tissue or microorganism	Reaction No.	Q$_{trans.}$	Reference
2. Plants			
Species and preparations:			
Chlorella (cells).....................	1b	0	23
	2b	0	23
Seeds, aqueous extracts; acetone-precipitated and dialyzed:			
Corn..............................	1b	1.4	2
	2b	0.8	2
	3a	0.4	2
Pea	1b	0.9	2
	2b	0.7	2
	3a	0.4	2
Seeds, aqueous extracts; $(NH_4)_2SO_4$-precipitated and dialyzed:			
Corn..............................	1b	1.5	2
	2b	0.0	2
	3a	1.2	2
Barley............................	1b	4.4	2
	2b	0.3	2
	3a	2.2	2
Pea...............................	1b	1.1	2
	2b	0.0	2
	3a	0.4	2
Clover............................	1b	1.7	2
	3a	1.6	2
Germinated seedlings, aqueous extracts; $(NH_4)_2SO_4$-precipitated and dialyzed:			
Corn..............................	1b	3.8	2
	2b	1.3	2
	3a	2.9	2
Pea...............................	1b	1.1	2
	2b	1.0	2
	3a	0.7	2
3. Bacteria and Yeast			
Brewers' yeast (aqueous extract)..............	1b	10	23
Bakers' yeast.............................	1b	0	23
	2b	0	23
Escherichia coli............................	1a	17	23

IV. Some Special Aspects of Transamination

1. Transamination in Cancer Tissue

In view of the fact that the transaminating enzyme systems act specifically on amino acids of the *l*-series, much emphasis was placed upon the observation (6) that amino group transfer is much slower in tumor tissue

than in normal tissue. Furthermore, it was reported (27) that reaction (2a) was not catalyzed by tumor tissue extracts. These observations were interpreted in the light of the reported presence of relatively large amounts of $d(-)$-glutamic acid in malignant tumor tissue (49). The thought was expressed that transamination studies might be of value in determining the malignancy of tumorous growths. However, with the accumulation of evidence to the effect that the glutamic acid present in tumor proteins has the normal l-configuration (17, 30, 31), the significance of the above results still lacks satisfactory explanation.

2. Transamination and Oxidative Deamination

Braunshtein and Bychov (8) have made the interesting suggestion that the transaminating enzymes may form a portion of the l-amino acid dehydrogenase system whose activity fails to survive destruction of the cell. In any case, they reported the successful oxidative deamination of amino acids of the l-series with cell-free enzyme solutions. A typical deamination was that of $l(+)$-alanine, brought about by the action of a cell-free solution containing glutamic aminopherase, glutamic dehydrogenase, and cozymase, together with $l(+)$-alanine, α-ketoglutaric acid, and an autoxidizable hydrogen carrier such as pyocyanine or methylene blue. The system formed about 12.5% of the theoretical amount of ammonia during three hours' incubation. The suggestion was offered that the l-amino acid dehydrogenase was not a simple enzyme system and thus did not survive extraction from the cell. Deamination was presumed to result from the combined action of at least two enzymes, together with suitable coenzymes and hydrogen carriers.

3. Transamination in Seeds and Plants

Virtanen and Laine (65) have ascribed a major role to transamination in the synthesis of amino acids and proteins in leguminous plants. They have presented evidence for their conception of the symbiotic fixation of nitrogen in leguminous root nodules, as outlined in Scheme 9. In addition, they presented evidence showing that $l(-)$-aspartic acid is the only α-amino acid formed by the root nodules, and suggested that all other amino acids of the plant proteins are formed by transamination reactions involving aspartic acid. The evidence given in support of this very comprehensive scheme is rather scant. A critical discussion has been published by Wilson (67).

Cedrangolo and Carandante (15) have studied transamination, with

$N_2 \longrightarrow$ hydroxylamine

oxime of oxalacetic acid

H_2

Carbohydrate $\longrightarrow$ oxalacetic acid $\qquad$ $l(-)$-aspartic acid

α-keto acids

oxalacetic acid $+$ l-amino acids
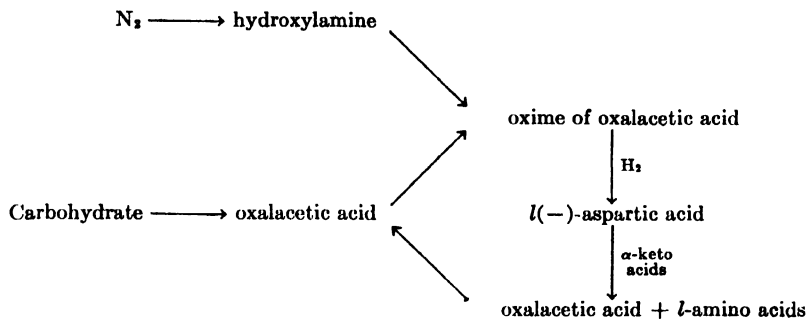
SCHEME 9

particular emphasis upon the rates of reactions (1), (2), and (3) in tissue extracts of a large variety of plants. Unfortunately, they employed rather unreliable analytical methods.

4. Transamination in Protein Synthesis

The role of transamination in protein synthesis and degradation has been the subject of much speculation and little work. Ågren (3) has reported that terminal amino groups of peptides such as glycyl-p-aminobenzoic acid and valylglycine can be transferred to α-ketoglutaric acid in the presence of minced cattle diaphragm muscle. He further states that alpha keto groups tied in peptide linkage to amino acids, *i. e.*, ketonic carbonyl groups adjacent to the carbonyl group of a peptide linkage, can act as amino group acceptors. A reaction analogous to the latter takes place in model transaminating systems (40) and has been discussed. Braunshtein and Bychov (8) failed to find peptides active as amino group donors in the presence of transaminating enzymes.

Linderstrøm-Lang (56) has suggested the series of reactions shown in Scheme 10, combining dehydrogenation and transamination in a hypo-

$$R'-\overset{\overset{O}{\|}}{C}-\overset{\overset{OH}{|}}{\underset{\underset{H}{|}}{C}}-OH + H-N-R'' \longrightarrow R'-\overset{\overset{O}{\|}}{C}-\overset{\overset{OH}{|}}{\underset{\underset{H}{|}}{C}}-\overset{\overset{}{\underset{H}{|}}}{N}-R'' \overset{-H_2}{\longrightarrow} R'-\overset{\overset{O}{\|}}{C}-\overset{\overset{OH}{|}}{C}=N-R''$$

$$\overset{+ \text{ glutamic acid}}{\longrightarrow} R'-\overset{\overset{NH_2}{|}}{\underset{\underset{H}{|}}{C}}-\overset{\overset{OH}{|}}{C}=N-R'' + \alpha\text{-ketoglutaric acid}$$
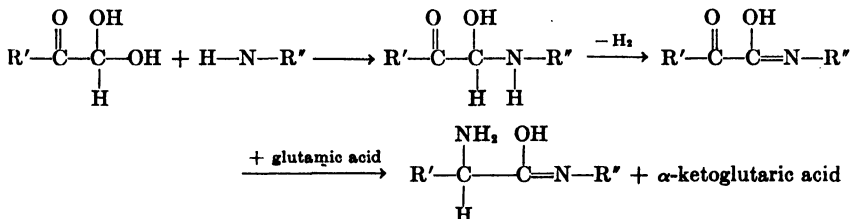
SCHEME 10

thetical peptide synthesis. The suggestion is based in part upon the work of Maurer and Woltersdorf (57) on the reaction between α-keto aldehydes and amines.

Schoenheimer and his collaborators (62) have speculated on the importance of amino group transfer from peptide chains and amino acids to suitable amino group acceptors with the formation of alpha keto acyl derivatives of peptides, and the reverse of this process. They have suggested that such transfers may account in part for the rapid entrance of dietary amino nitrogen, administered in the form of a single amino acid, into all the amino acids of the body proteins.

V. Comparison of Transamination in Model Systems and in Enzyme-Catalyzed Systems

The two types of transamination reactions discussed in the foregoing chapters have certain features in common. In both reactions amino groups are transferred from α-amino acids to α-keto acids without the intermediary formation of ammonia. Both reactions may be considered as coupled, intermolecular oxidation-reduction processes; and both reactions are rapid with certain compounds and slow with others.

The conditions under which the two systems function differ markedly. The model reaction proceeds most readily in acid aqueous solutions, and spontaneously at the boiling point of the solution. The enzyme-catalyzed reaction proceeds best at 37° C. and at pH 7.5 in aqueous solution.

The model reaction shows no specificity for configuration; optically active forms react at the same rate as the inactive forms. The products are always the optically inactive forms. The enzymatic system reacts preferentially with amino acids of the l-series, and the newly formed amino acids presumably belong to this series. The evidence concerning the latter point is sketchy, even though there is little doubt as to the results of proper experiments.

In model systems, monocarboxylic amino and keto acids react with each other readily; in fact, these reactions are examples of the most rapid types. The reaction proceeds most rapidly when the substituent on the alpha carbon atom of the amino acid, for instance the phenyl group, has a marked activating effect. Enzyme-catalyzed reactions proceed only if one member of a transaminating pair is a dicarboxylic amino or keto acid.

The decarboxylation observed in model systems does not occur in enzyme-catalyzed reactions. Thus the former are not reversible reactions, since one of the products is eliminated from the system, while the latter are reversible reactions and reach definite equilibrium points.

The mechanism of the two types of reactions is quite different. The model reactions proceed without the dissociation of a proton from the alpha carbon atom of the amino acid residue, a process which forms an important step in the enzyme-catalyzed reactions.

The enzyme-catalyzed reactions are closely simulated in model systems when the free carboxyl groups of the amino and keto acids in the model system are blocked by esterification. Under these conditions, the reaction takes place at lower temperatures and is influenced by catalysts such as sodium ethylate. No decarboxylation takes place and the reaction appears to be reversible.

It seems unfortunate that so much of the work upon enzyme-catalyzed transaminations has been done with crude analytical methods or with indirect analytical methods. This has led to results which often do not agree, and to confusing interpretations. The task of carrying out reactions on a sufficiently large scale to permit isolation of the products themselves, rather than the measurement of a few milliliters of nitrogen gas or of a few microliters of carbon dioxide, does not appear to be insurmountable now that sources of the enzyme are known to be easily available. Certainly the results would be less frequently contradictory.

Bibliography

1. Abderhalden, E., and Schmidt, H., *Z. physiol. Chem.*, **72**, 37 (1911).
2. Adler, E., Günther, G., and Everett, J. E., *ibid.*, **255**, 27 (1938).
3. Ågren, G., *Acta Physiol. Scand.*, **1**, 233 (1940).
4. Akasi, S., *J. Biochem. Japan*, **25**, 291 (1937).
5. Braunshtein, A. E., *Enzymologia*, **7**, 25 (1939).
6. Braunshtein, A. E., and Azarkh, R. M., *Nature*, **144**, 669 (1939).
7. Braunshtein, A. E., and Azarkh, R. M., *Biokhimiya*, **5**, 1 (1940).
8. Braunshtein, A. E., and Bychov, S. M., *Nature*, **144**, 751 (1939); *Biokhimiya*, **5**, 261 (1940).
9. Braunshtein, A. E., and Kritsman, M. G., *Biokhimiya*, **2**, 242 (1937); *Nature*, **140**, 503 (1937); *Biokhimiya*, **2**, 859 (1937); *Enzymologia*, **2**, 129 (1937).
10. Braunshtein, A. E., and Kritsman, M. G., *Biokhimiya*, **3**, 590 (1938).
11. Braunshtein, A. E., and Kritsman, M. G., *ibid.*, **4**, 303 (1939).
12. Breusch, F. L., *Biochem. J.*, **33**, 1757 (1939).
13. Brewer, S. D., and Herbst, R. M., *J. Org. Chem.*, **6**, 867 (1941).
14. Bychov, S. M., *Biokhimiya*, **4**, 189 (1939).
15. Cedrangolo, F., and Carandante, G., *Boll. soc. ital. biol. sper.*, **15**, 482 (1940).
16. Cherbuliez, E., and Herzenstein, A., *Helv. Chim. Acta*, **17**, 1440 (1934).
17. Chibnall, A. C., Rees, M. W., Tristram, G. R., Williams, E. F., and Boyland, E., *Nature*, **144**, 71 (1939).
18. Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
19. Cohen, P. P., *ibid.*, **33**, 1478 (1939).

20. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
21. Cohen, P. P., *ibid.*, **136**, 585 (1940).
22. Cohen, P. P., *Federation Proc.*, **1**, 273 (1942).
23. Cohen, P. P., *unpublished experiments.*
24. Cohen, P. P., and Hekhuis, G. L., *J. Biol. Chem.*, **140**, 711 (1941).
25. Cohen, P. P., and Hekhuis, G. L., *Cancer Research*, **1**, 620 (1941).
26. Cohen, P. P., Hekhuis, G. L., and Sober, E. K., *ibid.*, **2**, 405 (1942).
27. Euler, H. von, Günther, G., and Forsman, N., *Z. Krebsforsch.*, **49**, 46 (1939); *Naturwissenschaften*, **27**, 214 (1939). Euler, H. von, Hellström, H., and Günther, G., *Arkiv Kemi Mineral. Geol.*, **13B**, 6 (1939).
28. Euler, H. von, Hellström, H., Günther, G., Elliot, L., and Elliot, G., *Z. physiol. Chem.*, **259**, 201 (1939).
29. Franke, W., *Biochem. Z.*, **258**, 280 (1933).
30. Graf, S., *J. Biol. Chem.*, **130**, 13 (1939).
31. Graf, S., Rittenberg, D., and Foster, G. L., *ibid.*, **133**, 745 (1940).
32. Grassmann, W., and Arnim, K. von, *Ann.*, **509**, 288 (1934).
33. Harding, V. J., and Warneford, F. H. S., *J. Biol. Chem.*, **25**, 319 (1916).
34. Harvill, E. K., and Herbst, R. M., *J. Org. Chem.*, **9**, 21 (1944).
35. Herbst, R. M., *J. Am. Chem. Soc.*, **58**, 2239 (1936).
36. Herbst, R. M., *unpublished data from the author's laboratory.*
37. Herbst, R. M., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 32 (1938).
38. Herbst, R. M., and Engel, L. L., *J. Biol. Chem.*, **107**, 505 (1934).
39. Herbst, R. M., and Rittenberg, D., *J. Org. Chem.*, **8**, 380 (1943).
40. Herbst, R. M., and Shemin, D., *J. Biol. Chem.*, **147**, 541 (1943).
41. Holmberg, C., *Skand. Arch. Physiol.*, **68**, 1 (1934).
42. Hsü, S. K., Ingold, C. K., and Wilson, C. L., *J. Chem. Soc.*, **1935**, 1778.
43. Karrer, P., and Appenzeller, R., *Helv. Chim. Acta*, **25**, 595 (1942).
44. Karrer, P., Koenig, H., and Legler, R., *ibid.*, **24**, 127 (1941).
45. Karrer, P., Koenig, H., and Legler, R., *ibid.*, **24**, 861 (1941).
46. Karyagina, M. K., *Biokhimiya*, **4**, 168 (1939).
47. Kisch, B., *Biochem. Z.*, **242**, 1 (1931); **244**, 440 (1932); **247**, 371 (1932); **249**, 63 (1932).
48. Knoop, F., and Martius, C., *Z. physiol. Chem.*, **258**, 238 (1939).
49. Kögl, F., and Erxleben, H., *ibid.*, **258**, 57 (1939).
50. Konikova, A. S., Kritsman, M. G., and Teis, R. V., *Biokhimiya*, **7**, 86 (1942).
51. Kritsman, M. G., *ibid.*, **3**, 603 (1938).
52. Kritsman, M. G., *Enzymologia*, **5**, 44 (1938).
53. Kritsman, M. G., *Nature*, **143**, 603 (1939).
54. Kritsman, M. G., *Biokhimiya*, **4**, 667 (1939).
55. Langenbeck, W., *Ber.*, **60**, 930 (1927); **61**, 942 (1928); *Chem.-Ztg.*, **60**, 593 (1936); Langenbeck, W. and Gödde, O., *Ber.*, **70**, 669 (1937); Langenbeck, W., Weschky, L., and Gödde, O., *ibid.*, **70**, 672 (1937).
56. Linderstrøm-Lang, K., *Ann. Rev. Biochem.*, **8**, 49 (1939).
57. Maurer, K., and Woltersdorf, E. H., *Z. physiol. Chem.*, **254**, 18 (1938).
58. Needham, D. M., *Biochem. J.*, **24**, 208 (1930).
59. Neuberg, C., and Kobel, M., *Biochem. Z.*, **188**, 197 (1927).
60. Retinger, J. M., *J. Am. Chem. Soc.*, **39**, 1059 (1917).
61. Ruhemann, S., *J. Chem. Soc.*, **97**, 2025 (1910); **99**, 792, 1486 (1911).

62. Schoenheimer, R., Ratner, S., and Rittenberg, D., *Science*, **89**, 272 (1939); *J. Biol. Chem.*, **130**, 703 (1939).

63. Strecker, A., *Ann.*, **75**, 27 (1850).

64. Traube, W., *Ber.*, **44**, 3145 (1911).

65. Virtanen, A. I., and Laine, T., *Nature*, **141**, 748 (1938).

66. Vyshepan, E. D., *Biokhimiya*, **5**, 271 (1940).

67. Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation.* Univ. Wisconsin Press, Madison, 1940.

68. Zorn, K., *Z. physiol. Chem.*, **266**, 239 (1940).

TYROSINASE

By

J. M. NELSON and C. R. DAWSON

New York, N. Y.

CONTENTS

I. Introduction

No attempt has been made in the present review to cover all the litera-
ture relating to the enzyme, tyrosinase. Because of the confusion in
naming and recognizing the properties and functions of the various phenol
oxidizing enzymes, such an attempt would only produce a vast array of
conflicting and confusing statements and postulations. Rather, it is
the purpose of this review to present an interpretation of the enzyme and
its action, an interpretation which has developed slowly and hesitantly
during about ten years' experience in the field. For this reason, only
certain aspects of the historical side of the subject are covered. For a
more extensive survey of the history, the reader is referred to the excellent
reviews by Kastle (43), Chodat (23), Raper (70), Onslow (58), Sutter (80),
Oppenheimer (62), and Franke (33).

1. Discovery of the Enzyme

Interest in the chemistry of the oxidases dates back to 1856 when
Schoenbein (77) pointed out the presence in the mushroom, *Boletus luridus*,
of an oxygen-activating agent which brought about the aerobic oxidation
of certain material in the plant, thus giving rise to the blue pigment forma-
tion as the plant matured. The next step in the direction of growth of our
knowledge concerning oxidases was Yoshida's (89) showing the presence
of the enzyme, laccase, in the latex of the lac tree. Some years later,
Bertrand (12), following up Yoshida's study of laccase, found the enzyme
to be present in several varieties of mushrooms. Together with Bour-
quelot (20), his attention was attracted to certain varieties of mushrooms,
Russula foetens and *R. nigricans*, which, instead of turning blue as the
oxidation progressed, turned red, and finally dark brown or black. In
the case of the juice from *R. nigricans*, a crystalline substance separated.
When this material was placed in contact with the oxidase present in the
juice, aerobic oxidation set in, yielding first a red-colored solution and
finally a black product resembling melanin. This crystalline material was
later shown by Bertrand (13) to be the amino acid, tyrosine. Finding
laccase to have no action on tyrosine and the *R. nigricans* oxidase without
action on hydroquinone, while laccase is active toward this dihydric phenol,
convinced Bertrand they were dealing with a new oxidase, which he
named "tyrosinase." Continuing his study of the new oxidase, Bertrand
(14) found it catalyzed the aerobic oxidation of several other aromatic
monohydric phenols besides tyrosine, such as *p*-hydroxyphenyl ethyl
amine, *p*-hydroxyphenyl methyl amine, *p*-hydroxyphenyl amine, *p*-

hydroxyphenyl propionic acid, p-hydroxyphenyl acetic acid, p-cresol, and phenol. On the other hand, when the hydroxyl group was absent in the above compounds, the oxidase was then unable to bring about any oxidation.

Immediately subsequent to these earlier studies of the enzyme, interest became centered on the action of the enzyme on tyrosine and its role in melanin formation in connection with animal and plant pigmentation. The focusing of interest in this direction was due to the following facts: first, that tyrosinase was found to differ from the two previously known oxidizing enzymes, laccase and peroxidase, in that it catalyzed the oxidation of tyrosine, which the other two failed to do; and second, the oxidation product of tyrosine appeared to be melanin, or a product closely resembling it. Bougault (19) and Harlay (40) recommended the use of the enzyme for the detection of the presence of tyrosine in biological products.

2. Occurrence of Tyrosinase

Tyrosinase occurs widely spread in nature, especially in plants and in invertebrates, but its presence in mammals has not been so easy to demonstrate. Onslow (59) reported its extraction from the skins of rabbits of various colored races. Certain pigmentations in plants and animals have been attributed to tyrosinase action (85). Gessard (34) showed the presence of the enzyme in the ink sac of the cuttlefish. Pinhey (64) reported the presence of tyrosinase in the blood of certain crustacea. Bhagvat and Richter (15) found evidence of the enzyme in the blood of arthropods. Arnow (8) claims that tanning of the skin by sunlight involves tyrosinase action. For a more complete reference concerning the occurrence of the enzyme in nature see Kastle's review (43).

Szent-Györgyi and Vietorisz (82) have suggested that the enzyme serves to form a protective coating over injured parts in plants. The oxidase is supposed to occur dormant in the plant tissue and becomes active when the tissue is injured. At such time, it brings about the oxidation of phenolic bodies present in the plant to quinones, and the latter in turn not only act in a bactericidal capacity but also combine with protein, forming an insoluble coating over the injured tissue.

Bodine et al. (4) have published during the past few years a series of papers on their study of an inactive form of tyrosinase, termed by them protyrosinase, which is formed after the diapause stage in the eggs of grasshoppers, *Melanoplus differentialis*, and in the larvae of the meal worm, *Tenebrio molitor*. Diapause eggs were triturated in 0.9% sodium chloride solution, centrifuged, the lipoidal layer removed, and the fluid brought to pH 6.8. After fractionating with ammonium sulfate, a precipitate was obtained

which was dissolved in 0.9% sodium chloride and dialyzed against the same concentration of sodium chloride. The resulting solution contained the inactive form of tyrosinase but no active tyrosinase. This protyrosinase was rendered active toward substrates such as tyramine and tyrosine by adding to the enzyme solution detergents, such as sodium oleate, aerosol (sodium lauryl sulfonate), chloroform, or acetone. It was also found (6) that shaking a solution of the protyrosinase resulted in irreversibly changing the latter into definite proportions of protyrosinase, active tyrosinase, and inactive products. Bodine and Tahmisian (16) also report that salts of mercury, gold, platinum, and palladium tend, when present within definite concentrations, to change protyrosinase into the active enzyme. Higher concentrations, however, proved toxic to the active enzyme but not to the protyrosinase. These investigators (7) also found that the protyrosinase begins to form in the grasshopper egg immediately following the diapause period according to a simple autocatalytic reaction.

The sources mostly used for obtaining preparations of tyrosinase, and for a study of the enzyme and its action have been the potato and the common mushroom, *Psalliota campestris*. The larvae of the meal worm, *Tenebrio molitor*, the wild mushroom, *Lactarius piperatus* (27), the blood of the octopus (22), the Indian bean (54), and wheat bran (14) have also been used.

In the natural state, tyrosinase is found accompanied by other phenol oxidases, peroxidases, catalases, etc., many of which tend to influence or modify its enzymatic action. Besides catalyzing the aerobic oxidation of many monohydric phenols, it has in common with the laccases and peroxidases the ability to bring about the oxidation of many *o*-dihydric phenols such as catechol, pyrogallol, etc. It is therefore distinguished from the other phenol oxidases by its ability to catalyze two essentially different oxidations, the insertion of an hydroxyl group into monohydric phenols ortho to the one already present, and the oxidation of many *o*-dihydric phenols to their corresponding *o*-quinones.

3. Early Views Concerning the Enzyme's Mode of Action

In the light of Schoenbein's early studies, involving activated oxygen and peroxide formation, it is not surprising that the earlier investigators of oxidase action considered it to depend on peroxide formation.

Among the first to propose a theory for the mechanism were Bach and Chodat (9, 23, 43). According to their view, plant tissues which darken on injury contain a substance, termed oxygenase, which in the presence of air suffers autoxidation, yielding a peroxide. This peroxide is then acted on by peroxidase, present in most plants, yielding active oxygen, which brings about the oxidation of the phenolic bodies also present in the plant tissue.

$$\text{Autoxidizable substance} + O_2 \longrightarrow \text{peroxide}$$

$$\text{Peroxide} + \text{phenolic body} \xrightarrow{\text{peroxidase}} \text{oxidized phenol} + \text{autoxidizable substance}$$
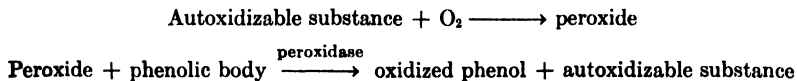
Onslow (57), having become interested in the darkening shown by many plant tissues, especially when injured, found that these plants contain o-dihydric phenol substances such as catechol, protocatechuic and caffeic acids. She also found that, when neutral aqueous solutions of the dihydric phenols are permitted to stand in contact with air for several days, they gradually turn brown. The fact that the darkening action was observed to be much faster in the plant tissue, and did not occur when the plant tissue had been boiled, led her to the conclusion that an enzyme was present in these plant tissues which catalyzed the autoxidation of the o-dihydric phenols. She therefore modified the Bach and Chodat concept of oxygenase by regarding the latter as being an enzyme instead of an autoxidizable substance, an enzyme which, in the presence of air, catalyzed the oxidation of the o-dihydric phenols, yielding as one of the oxidation products a peroxide. The peroxide in the presence of peroxidase, which is present in most plant tissues, was considered to produce active oxygen, and the latter could oxidize both o-dihydric and monohydric phenols.

In 1925 Szent-Györgyi (81) succeeded in showing that this darkening of plant tissues could take place in the absence of peroxidase. This led Onslow to modify her theory concerning the enzymatic oxidation of the monohydric phenols. She suggested that the introduction of the second hydroxyl group into the monohydric phenols was most likely brought about directly by the o-quinones or by the hydrogen peroxide formed when the o-dihydric phenols were oxidized by means of the oxygenase.

About this time, Raper and coworkers were engaged in unraveling the intermediate reactions involved in the conversion of tyrosine into melanin by tyrosinase. In connection with this study, they succeeded (70) in establishing the fact that tyrosinase possessed the ability to bring about, either directly or indirectly, the insertion of a new hydroxyl group in the ortho position to the one already present in a monohydric phenol and then to catalyze the oxidation of the catechol compound thus formed to an o-quinone. Pugh and Raper (68) therefore concluded that "tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative.... There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow."

In criticizing this conclusion of Pugh and Raper, Onslow and Robinson (61), in the following year, agreed that all plant extracts exhibiting tyrosinase activity likewise exhibited oxygenase activity, that is, all extracts possessing the ability to catalyze the aerobic oxidation of monohydric phenols such as tyrosine or p-cresol could likewise catalyze the oxidation of catechol compounds. However, the reverse was not always true; furthermore they were able to demonstrate that, in extracts possessing both activities, the monohydric phenol activity (tyrosinase) could be removed by charcoal treatment and the oxygenase action still retained. They also demonstrated that traces of catechol greatly accelerated the monohydric phenol oxidation, and suggested that tyrosinase was oxygenase intimately associated with an o-dihydric phenol or its derived o-quinone; they held that monohydric phenol action is indirect, in the sense that oxidation products of catechol, "probably o-quinones and hydrogen peroxide," were involved in the conversion of monohydric phenols to their corresponding o-dihydric phenols. They could see no logic, therefore, in substituting the name tyrosinase for an enzyme which "primarily catalyzes the oxidation of substances with the o-dihydroxy grouping." The two views may be summarized as follows:

1. Onslow's oxygenase catalyzes the oxidation of o-dihydric phenols; and the oxida-

tion of the monohydric phenols to the *o*-dihydric condition is brought about by products formed in the enzymatic oxidation of the *o*-dihydric phenols.

2. Raper's view, on the other hand, regards the enzyme as catalyzing the oxidation of both the monohydric and *o*-dihydric phenols.

II. Tyrosinase Preparations

Although crude tyrosinase preparations may be obtained from a number of different sources, experience has shown that the purified enzyme can most easily be prepared from sources particularly rich in the enzyme, such as the common edible mushroom, *Psalliota campestris*, or the ordinary potato, *Solanum tuberosum*. The mushroom is the better of these two sources, for aqueous extracts of the potato contain relatively large amounts of inert globulinlike proteins difficult to remove from the enzyme. For this reason, most of the purified tyrosinase preparations studied in the authors' laboratory have been obtained from the mushroom.

The methods employed for isolating and purifying the enzyme from the crude expressed juices or aqueous extracts are those of the protein chemist and involve operations such as fractional salt precipitations, fractional precipitations with alcohol or acetone, adsorption to agents such as alumina and calcium phosphate gel, elution, precipitation with lead acetate, dialysis, etc. Throughout these operations, the enzyme behaves in all respects like a water-soluble protein. It is very sensitive to elevated temperatures and low pH, and all operations must be carried out above pH 5 and in the vicinity of room temperature or lower to insure a respectable yield of the enzyme. The expressed juices or aqueous extracts of the mushroom or potato darken very rapidly on exposure to air. During this process, enzyme activity is frequently lost. It is advisable, therefore, to remove as much as possible of the natural phenolic substrate (or substrates) at an early stage in the purification. Experience has shown that a cold acetone wash (dry-ice chilled) of the ground tissue before extraction with water or dilute salt solution helps to yield colorless enzyme preparations. The fact that the enzyme is generally quite unstable in the crude extract and becomes more stable after partial removal of accompanying protein material by fractional salt precipitations, etc., suggests that inactivating agents, possibly proteolytic enzymes, may be present in the crude extract along with the tyrosinase. It is generally advisable, therefore, to carry out the first few stages in the purification of the extract within the same day. Crude extracts appear to be considerably more stable when allowed to stand at pH 9 to 10 than at pH 5 to 7; but they develop more color at the elevated pH and the color is very difficult to remove in subsequent purification operations.

Various methods have been proposed for following the activity of tyrosinase during the isolation, purification, and concentration of the enzyme. These methods are discussed in more detail in Sections IV and VI. However, it should be emphasized again that the crude juices or aqueous extracts possess the ability to catalyze the aerobic oxidation of both monohydric and o-dihydric phenols. It has become the practice to use p-cresol and catechol as experimental substrates for following these two activities. In the crude juice or extract the ratio of these two activities (catechol: p-cresol) is generally in the region of 1:1 to 5:1, depending on the method used for measuring the catecholase activity. During the process of purification, this ratio can be, and generally is, greatly increased. The resulting "purified" enzyme preparation, therefore, is frequently relatively free of monophenolase action. This fact has led workers in the field (44, 45) to abandon the early name, tyrosinase, for the crude phenol-oxidizing enzyme, and use the name polyphenol oxidase, a name which has found wide adoption by other workers in the field.

This tendency to ignore the monohydric phenol action of the enzyme is an unsatisfactory way out of a problem that has perplexed many workers in the field, and, as already pointed out in the introductory part of this review, has not gone entirely without challenge. At first glance, there would seem to be little ground for criticism, for the pronounced change in ratio of the two activities observed during the process of purification would normally suggest a separation of two enzymes, or one enzyme plus some unknown factor. However, during the past five years, considerable evidence that the two activities belong to the same enzyme complex has been accumulated. By altering the procedure of preparation somewhat, highly purified enzyme solutions have been obtained in which the ratio of the two activities was found to be quite comparable to that found in the crude juice or extract. Before discussing this point any further, it seems advisable to discuss the role that copper plays in the enzyme action.

1. The Role of Copper

While purifying the phenol oxidase of the potato, Kubowitz (45), in 1937, found a linear relationship between the copper content of his preparations and the catalytic activity (toward catechol) of the enzyme. His best preparation had a copper content of 0.2% and a Q_{O_2} of 34,500.

The term Q_{O_2} is a measure of the activity and purity of the enzyme in that it expresses in mm.^{3} of oxygen uptake the rate of oxidation of the substrate (catechol) per hour per milligram of enzyme.

The purified enzyme catalyzed the oxidation of o-dihydric phenols such as catechol, pyrogallol, 3,4-dihydroxyphenylalanine, caffeic acid, protocate-chuic acid, adrenaline, etc., but had no direct action on resorcinol, hydroquinone, or ascorbic acid. The latter two compounds could be oxidized indirectly, however, by adding a small amount of catechol to the system. Monohydric phenols such as phenol and p-cresol were oxidized only relatively slowly after a lag period. Tyrosine was oxidized directly, without any evidence of a lag period, but at a rate calculated to be about 1100 times slower than catechol. The latter was found to be oxidized the most rapidly of all substrates tried.

Kubowitz found that the enzyme activity was inhibited by agents which chemically combine with copper, such as cyanide, diethyldithiocarbamate, salicylaldoxime, and carbon monoxide. He proved that the copper was an essential part of the enzyme when he split it off by a treatment involving cyanide and dialysis, and then demonstrated that the copper-free protein no longer possessed the ability to catalyze the oxidation of catechol. He found that, just as in the case of the hemocyanin copper proteins, the resolution was reversible. Practically complete enzyme activity could be restored by adding excess copper ion to the inactive protein. Other divalent metals such as iron, cobalt, nickel, manganese, and zinc were found to be ineffective in restoring the enzyme activity when added to the copper-free inactive protein.

Allen and Bodine (5) have also shown that copper is an essential constituent of protyrosinase from grasshopper eggs. Protyrosinase from which the copper had been removed by a method similar to that used by Kubowitz could not be converted into an active tyrosinase. The ability to convert the inactive protyrosinase into active enzyme by the use of Aerosol was restored by adding excess copper sulfate to the copper-free protyrosinase. Just as found by Kubowitz, salts of iron, cobalt, nickel, manganese, and zinc were unable to replace those of copper. Tenenbaum and Jensen (84) have recently reported that under certain conditions salts of iron, cobalt, and manganese, as well as those of copper, can "reactivate" mushroom tyrosinase which has been "inactivated" by potassium cyanide, sodium diethyldithiocarbamate, or potassium ethyl xanthate

As the result of further comparison of the potato oxidase with hemocyanin, particularly in respect to carbon monoxide inhibition studies, Kubowitz concluded that the copper in the enzyme as isolated is in the cupric state. He postulated that, in its reaction with catechol, the enzyme copper is reduced to the cuprous state, which in turn is reoxidized by molecular oxygen. The two-stage reaction may be represented as:

$$4Cu^{++} \text{ (enzyme)} + 2 \text{ catechol} \longrightarrow 4Cu^{+} \text{ (enzyme)} + 2 \text{ } o\text{-quinone} + 4H^{+} \quad (1)$$

$$4Cu^{+} \text{ (enzyme)} + 4H^{+} + O_2 \longrightarrow 4Cu^{++} \text{ (enzyme)} + 2H_2O \quad (2)$$
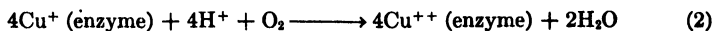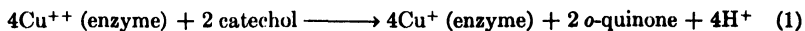
The following year, Keilin and Mann (44) found that the phenol oxidase of the cultivated mushroom, *Agaricus* or *Psalliota campestris*, was also a copper–protein compound. They isolated, in highly purified form, about 10 mg. of the enzyme from 15 kg. of fresh mushrooms. Their best preparation contained 0.3% copper and had a Q_{O_2} of 1,160,000 as measured on catechol. On the basis of the copper content, as compared with that of various crystalline hemocyanins, they considered the enzyme to be pure. They emphasized the fact that the polyphenolase activity of the enzyme (oxidation of catechol or pyrogallol) became proportional to the copper content only after considerable purification. In other words, much of the copper initially present in the mushroom was found to have no relationship to the enzyme. The purified enzyme was found to be completely free from hematin, thereby weakening the suggestion of Yakushiji (88) that a hematin compound constitutes the active grouping of polyphenol oxidase from mushrooms (*Lactarius piperatus*). (Dalton and Nelson (27) found no iron in highly purified tyrosinase from *L. piperatus*.) All the oxidation reactions which were catalyzed by the purified enzyme, either directly or indirectly, were found to be strongly inhibited by potassium cyanide, hydrogen sulfide, and carbon monoxide.

Keilin and Mann also emphasized that, whereas crude extracts of the mushroom readily oxidized monohydric phenols such as *p*-cresol, this property was rapidly lost during the purification, and the purified enzyme became highly specific for the oxidation of *o*-dihydric phenols such as catechol and pyrogallol. They called the enzyme, therefore, polyphenol oxidase, and dismissed the monophenolase activity with the statement that it "probably requires the presence or the gradual formation of an additional factor, the nature of which remains still to be determined."

2. High Catecholase and High Cresolase Preparations

For reasons which have been indicated previously, and which will become more apparent later in this review, it has become the practice in the authors' laboratory to refer to purified enzyme preparations possessing marked activity toward catechol (catecholase activity) and little activity toward *p*-cresol (cresolase activity) as high catecholase preparations of tyrosinase. Ludwig and Nelson (46) and Parkinson and Nelson (63) reported on thirteen different preparations of this type from the mushroom, *Psalliota campestris*, and found, as did Keilin and Mann, that the catecholase activity became proportional to the copper content of the enzyme after several steps in the purification. Their best preparations ran about 0.1% in copper and had a catecholase activity of about 350

units per γ of copper, or per mg. dry weight of enzyme. On the basis of the copper content and Q_{O_2} calculated from their units, these preparations were approximately one-third as pure as the best preparation of Keilin and Mann (0.3% copper, $Q_{O_2} = 1,160,000$).

The catechol–hydroquinone unit of Adams and Nelson (3) was used. This unit is defined as the amount of enzyme that will catalyze the oxygen uptake of 10 mm.[3] per minute while oxidizing a substrate mixture of catechol and hydroquinone at 25° C. Therefore the calculated $Q_{O_2} = 10 \times 60 \times 350 = 210,000$. For this type of preparation (high catecholase), the activity obtained using a mixture of catechol and hydroquinone is smaller by a factor of about 2 than that obtained using catechol directly (37). Thus a corrected value of 700 units per γ copper or per mg. enzyme ($Q_{O_2} = 420,000$) should be used to compare with the Q_{O_2} value of Keilin and Mann.

More recently, preparations of the above type having a much higher degree of purity have been obtained in the authors' laboratory. As nearly as can be judged, they are very similar to the polyphenol oxidase of Keilin and Mann. They will be discussed in Section VIII.

TABLE I

AVERAGE VALUES FOR ACTIVITY OF TYROSINASE PREPARATIONS*

Enzyme type	Cu, %	Ratio of catecholase to cresolase	Activity			
			Units per mg.		Units per γ Cu	
			Cat.	Cres.	Cat.	Cres.
High catecholase	0.1	High and variable	700	Low and variable	700	Low and variable
High cresolase	0.1	2	350	175	350	175

* Showing average values obtained by Parkinson and Nelson (63) using a dozen or more different high catecholase and high cresolase preparations of tyrosinase. All catecholase activity values in this table are those of the direct oxygen uptake method on catechol (see discussion at top of this page). The activity values in units when multiplied by 600 give Q_{O_2} values.

As previously pointed out, it is possible, as shown by Parkinson and Nelson (63), to prepare from the same source, the common mushroom, purified enzyme in which the ratio of the two activities is quite comparable to that found in the expressed juice or crude extract. Since these purified preparations are relatively high in monophenolase activity as compared with the more easily obtainable and therefore more common high catecholase type of enzyme, they have been called high cresolase preparations of tyrosinase. Parkinson and Nelson made a large number of preparations of this type and showed that both activities were proportional to the

copper content of the purified enzyme. The best preparations contained about 0.1% copper and possessed an activity of 175 cresolase units per γ of copper or per mg. dry weight. (The cresolase unit of Adams and Nelson (2) was used. This unit is defined in Section IV.) All the preparations showing this proportionality factor between cresolase activity and copper likewise exhibited a constant catecholase to cresolase activity ratio of about 2, *i. e.*, about the same as found in the crude plant juice or extract. These facts led Parkinson and Nelson to conclude that "tyrosinase as it exists in the common mushroom is one enzyme complex rather than two separate independent factors." From the activity ratio it follows that the catecholase activity per γ of copper for the high cresolase enzyme was found to be about one-half that of the high catecholase enzyme. These points of comparison are clarified by the data shown in Table I.

3. Tyrosinase from the Wild Mushroom

In 1939, Dalton and Nelson (27) isolated from the wild mushroom, *Lactarius piperatus*, a highly purified tyrosinase preparation that had as its most striking characteristic a high monophenolase activity (*p*-cresol) as compared with its activity toward catechol. Throughout the process of isolation and purification, the activity ratio (*p*-cresol : catechol) was about 10:1 and could not be altered. In this respect, therefore, it differed markedly from the enzyme as isolated from the common mushroom, *Psalliota campestris*. Methods of purification which experience had shown would cause a marked change in the activity ratio of the enzyme as isolated from *P. campestris* were without effect in this case. Dalton and Nelson found that both activities became proportional to the copper content after several steps in the purification process. The purest enzyme preparation obtained contained 0.23% copper, possessed an activity of 1000 cresolase units per mg. dry weight and 370 cresolase units per γ of copper; Q_{O_2} (*p*-cresol) = 600,000; Q_{O_2} (catechol) = 60,000.

It should be emphasized that Dalton and Nelson used the catechol–hydroquinone unit (3) of catecholase activity. It is now recognized that hydroquinone acts as an inhibitor, and all catecholase activity measurements made on a catechol–hydroquinone substrate mixture are considerably lower than those made on catechol directly (see discussion on page 108). It seems probable, in view of the later experience of Parkinson and Nelson with high cresolase preparations of tyrosinase from *P. campestris* that, had Dalton and Nelson measured the catecholase activity on catechol rather than a catechol–hydroquinone mixture, they would have recorded a much higher catecholase activity for the tyrosinase from *L. piperatus*. In other words, the activity ratio (*p*-cresol:catechol) would have still been constant but much lower than 10:1—possibly in the region of 1:1 or 1:2 as found by Parkinson and Nelson (63).

The purified enzyme contained no iron, and concentrated solutions were practically colorless. Two absorption bands were observable in the ultraviolet region of the spectrum: a pronounced band due to protein at 273 mμ and a weak band at about 330 mμ probably due to copper.

Colorless, well-shaped crystals belonging to the isometric system were obtained from rather crude as well as from purified preparations of the tyrosinase. The crystals contained 0.25% copper, 13.6% nitrogen, and in solution showed the same ultraviolet absorption as did the purified enzyme solution. The procedure followed in obtaining the crystals was similar to that usually employed for crystallizing hemocyanin, *i. e.*, by adjusting the pH of the enzyme solutions, containing at least 1000 cresolase units per ml., to about 4.8 by means of sodium acetate buffer, and then adding ammonium sulfate until the solution became slightly cloudy. After standing for several hours, crystals could be observed. In no case, however, was more than 10% of the dry weight of a purified enzyme preparation obtained in crystalline form.

The crystals were insoluble in water, dilute acids, and salt solutions, but when freshly prepared were soluble in dilute alkali (secondary sodium phosphate solution, pH 8). They became increasingly insoluble on standing and, after a few weeks, became practically insoluble in 0.1 *M* sodium hydroxide. Solutions of the crystals were only slightly active toward p-cresol and catechol; but, on standing, such solutions showed a tendency to increase in activity. The ratio of the activities in these solutions of the crystalline material was also 10:1. Dalton and Nelson concluded that the enzyme and the crystals were very closely related:

"Either the enzyme becomes, for some unknown reason, inactive when it is crystallized, or an inactive form, such as a proenzyme, accompanies the enzyme as it is purified."

III. The Enzymatic Oxidation of Catechol

As previously indicated, two substrates have been widely used for studying the mode of action of tyrosinase, *i. e.*, p-cresol for the monohydric phenol activity and catechol for the o-dihydric phenol activity. Since the mechanism of the oxidation of the o-dihydric phenol seems to be the simpler, it will be considered first.

The complete enzymatic oxidation of catechol as followed by manometric measurements of oxygen absorption requires the uptake of two gram atoms of oxygen per mole of catechol (68, 73). In 1925, Szent-Györgyi (81) and Happold and Raper (39) independently suggested that o-benzoquinone was formed in the reaction, and this view was accepted by other workers

in the field (60). The following year, Pugh and Raper (68) produced supporting experimental evidence when they enzymatically oxidized catechol in the presence of aniline, and isolated the dianilinoquinone complex. The oxidation of catechol to o-benzoquinone stoichiometrically requires, however, only one atom of oxygen, and thus much of the interest in the mechanism of the oxidation has been centered on the fate of the second atom of oxygen uptake.

1. The Hydrogen Peroxide Question

It has long been recognized that the slow autoxidation of many substances, including phenolic bodies, results in the formation of hydrogen peroxide (49). It is not surprising, therefore, that one of the first mechanisms for the enzymatic oxidation of catechol, as proposed by Onslow and Robinson (60), accounted for the second atom of oxygen by the formation of hydrogen peroxide. They looked upon the enzyme as a catalyst for the autoxidation of catechol, that is:

$$C_6H_4(OH)_2 + O_2 \xrightarrow{\text{enzyme catalyst}} C_6H_4O_2 + H_2O_2 \qquad (3)$$
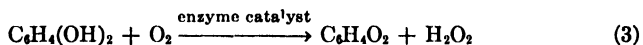
and attributed the great difficulty they experienced in detecting traces of hydrogen peroxide among the reaction products of the enzymatic oxidation to the fact that their enzyme preparations contained catalase and peroxidase. Platt and Wormall (65) supported this view, but Pugh and Raper (68) and later Nobutani (55) expressed doubt concerning the formation of hydrogen peroxide during the enzymatic oxidation of catechol, pointing out that the catalase present in their tyrosinase preparations would decompose any hydrogen peroxide as rapidly as formed. This would return an atom of oxygen to the system and the net result would be an oxygen uptake of one atom instead of two as actually observed.

Nevertheless, the above mechanism, accounting for the second atom of oxygen uptake by means of hydrogen peroxide formation, met with favor and remained prominent for several years. This was because it seemed to help explain several peculiarities of tyrosinase action. For example, it was used to explain the marked inactivation of the enzyme that is readily apparent during the enzymatic oxidation of catechol, for experience had shown that hydrogen peroxide has a destructive action on many enzymes. Likewise, it was used to explain the action of tyrosinase in converting monophenols into catechol (67), since it had been known for a long time that hydrogen peroxide in the presence of certain catalytic agents such as ferrous salts would convert benzene into phenol, phenol

into catechol, etc. (25, 48). Raper (69) showed that tyrosine could be converted to 3,4-dihydroxyphenylalanine by similar means. However, Bordner and Nelson (17) later demonstrated that hydrogen peroxide was not involved in the action of tyrosinase on monophenols (see Section VI).

In 1938, Dawson and Nelson (30) developed a method for following the enzymatic oxidation of catechol by means of titrating the iodine liberated from acidified potassium iodide solutions by the products of the enzymatic reaction. They found that, when the catechol was rapidly oxidized in dilute solutions, buffered within the region of pH 4 to pH 7, the iodine titer corresponded to that of o-benzoquinone, i. e., two equivalents of iodine were liberated for every molecule of catechol oxidized. Furthermore, the stability properties of the initial product were similar to those of o-benzoquinone prepared from catechol by the action of ceric sulfate (29). It became apparent that the mechanism involving hydrogen peroxide formation during the enzymatic reaction, as postulated by Onslow and Robinson (Eq. 3), could not be correct. Using tyrosinase preparations which had been purified until free of peroxidase and catalase activity, Dawson and Ludwig (28) showed that the oxidation product became much less stable as soon as hydrogen peroxide was added to the system. Only in the region of pH 4 or lower could one expect to find a reasonably stable oxidation product made up of o-benzoquinone and hydrogen peroxide. When large amounts of catalase and peroxidase were added to the system during the enzymatic oxidation of catechol at this pH, no effect could be observed on the course of the oxidation as followed by the iodometric titration method. Dawson and Ludwig concluded:

"The consumption of two atoms of oxygen per molecule of catechol during the enzymatic oxidation cannot be directly attributed to the formation of hydrogen peroxide."

2. The Second Atom of Oxygen

The rejection of the hydrogen peroxide mechanism (Eq. 3) stimulated interest again in the role of the second atom of oxygen uptake always observed during the complete enzymatic oxidation of catechol. Pugh and Raper (68) were among the first to suggest that the second atom was involved in a further oxidation of the o-benzoquinone, the nature of the reaction and the higher oxidation product being unknown to them. Dawson and Nelson (29) pointed out that the kinetics of the disappearance of o-benzoquinone in dilute solutions indicated a simple hydration reaction between the quinone and water, the rate of which increased rapidly with an increase in pH, being relatively slow at pH 4. Following the enzymatic

oxidation by oxygen-uptake measurements, they were able to demonstrate a two-stage oxidation using dilute solutions of catechol buffered to pH 5. A marked decrease in rate occurred in the oxygen-uptake curve at the one-atom stage, indicating that the uptake of the second atom of oxygen had to await some change in the initial oxidation product (30).

When Wagreich and Nelson (86) correlated the iodometric titration method with the oxygen-absorption method of following the enzymatic oxidation of catechol, several new facts came to light. The initial oxidation product as determined by iodometric titration corresponded exactly to one atom of oxygen uptake, thereby confirming the belief that it was o-benzoquinone. Of more interest was the observation that, during the disappearance of o-benzoquinone in aqueous solutions, a new substance, behaving in all respects toward the enzyme like catechol, was formed. The quinone–water reaction was found to be pseudomonomolecular in character; and two molecules of the quinone led to the formation of one molecule of catechol. It appeared likely from previous studies (87) that the other product of the quinone–water reaction was an hydroxy derivative of o-benzoquinone (probably 4-hydroxy), that is:

(4)
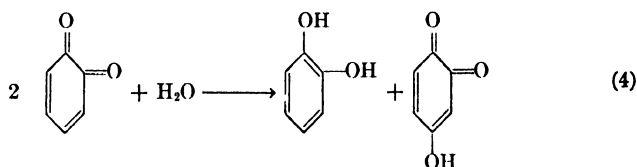
3. Mechanism of the Oxidation

All the evidence now available indicates that the enzymatic oxidation of catechol in dilute solutions buffered between pH 4 and 7 proceeds through a series of consecutive reactions:
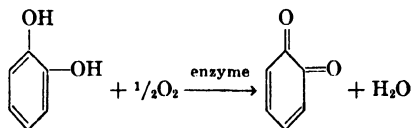
COMMENT

(5a) Fast; rate dependent on amount of enzyme
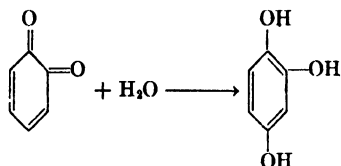
(5b) Rate dependent on pH and concentration. Relatively fast at pH 7, slow at pH 4

$$(5c) \quad \text{Probab y} \\ \text{instantaneous}$$

$$(5d) \quad \text{Same as } (5a)$$

SUM:

$$(5)$$

$$(6)$$

Highly colored
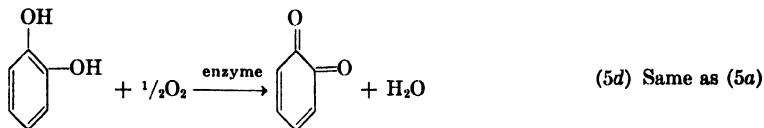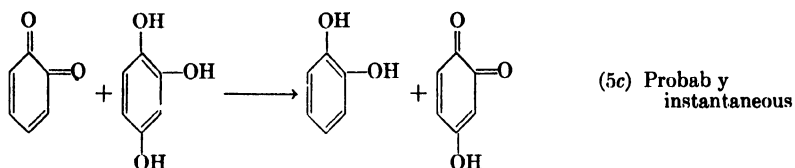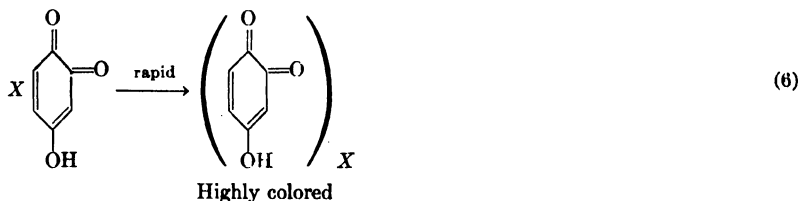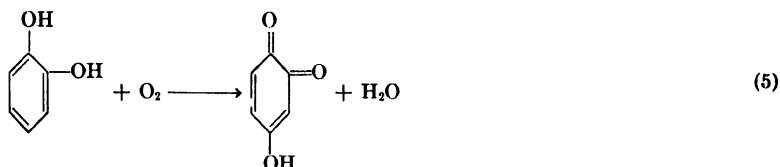
The sum of these consecutive reactions (a, b, c, d) is the over-all reaction (Eq. 5), which accounts for the two atoms of oxygen uptake per mole of catechol oxidized. It is believed that the hydroxyquinone rapidly polymerizes (Eq. 6) to yield the dark-colored humic-acid-like pigments (71). The quinone reactions in this mechanism are similar to those proposed by Fieser and Peters (32) to account for the disappearance of β-naphthoquinone in aqueous solutions.

The above mechanism of consecutive reactions explains why a change in rate of oxidation, as measured by oxygen uptake, occurs at the one-atom stage when very dilute solutions of catechol are enzymatically oxidized at relatively low pH. The second atom of oxygen uptake (Eq. 5d) must then await the water reaction (Eq. 5b). The mechanism is undoubtedly more complicated when relatively concentrated solutions of catechol are used. There is evidence (29, 32, 81) that catechol reacts with o-benzoquinone; and it seems possible that it would occur in a manner similar to the water reaction (Eq. 5b).

IV. The Measurement of Tyrosinase Activity

Before any success can be obtained, or hoped for, in isolating a physiologically active principle such as an enzyme, etc., from a natural source such as plant or animal tissue, it is necessary first to develop a reliable means of following the principle during its isolation, purification, and concentration. The activity measurement for this purpose, usually a rate measurement, may or may not be reliable, depending on whether or not optimum conditions for the measurement have first been established. Activity measurements made under nonoptimal conditions may be worse than none, for they can be entirely misleading. For this reason, it is first advisable to learn in some detail the effect on the activity determination of variation in experimental conditions of measurement. In any case, the activity or rate measurement by itself is of no value as a criterion of purity. To serve this purpose it must be correlated with some other factor. In the case of enzymes, the activity is usually correlated with the dry weight of nondialyzable material, or the per cent nitrogen in the nondialyzable material.

It must also be kept in mind, particularly in the case of certain enzymes, that the activity determination frequently is carried out on a substrate that may not be the natural one, i. e., the substrate which occurs with the enzyme in the plant or animal tissue. Many times the complete identity of the natural substrate is unknown and the selection of the experimental substrate has been based on trial-and-error studies of the specificity characteristics of the crude enzyme. These specificity characteristics may change during the process of isolation and purification of the enzyme, for they may be controlled by other protein or nonprotein factors present in the crude enzyme, or the enzyme protein itself may be chemically altered during the process.

It is of interest to consider the case of tyrosinase in this light. As previously pointed out, Bertrand (13) gave the enzyme its name when he found that the amino acid tyrosine, on which the enzyme was active, occurred with it in the juices of the mushroom, *Russula nigricans*. Early workers used tyrosine as the experimental substrate for assaying the enzyme; and several methods have been described (24, 38) based on colorimetric measurements of amounts of melanin formed, or upon determinations of unoxidized tyrosine remaining. Raper (70) was among the first to use the Warburg respirometer as a means of quantitatively following oxygen absorption during the oxidation of tyrosine, but he was more interested in working out the chemistry of the oxidation rather than developing a method of enzyme assay. Raper's work showed that the enzymatic oxidation of tyrosine involved both monophenol and o-dihydric phenol oxidation and was further complicated by secondary reactions involving the amino group. Factors such as these, coupled with the low solubility of tyrosine and the greater

activity of the enzyme with simpler phenols, led to the use of more easily available and less complex phenols for enzyme assay. *p*-Cresol and catechol have been widely used for the purpose.

The earlier colorimetric methods of enzyme assay were not very satisfactory from a quantitative viewpoint, and have been largely displaced by manometric methods based on measuring the rate of oxygen consumption during aerobic oxidation of the phenolic substrate as catalyzed by the enzyme. The rate of pigmentation during the enzymatic reaction is not necessarily proportional to the rate of oxidation, *i. e.*, to the enzyme, for the pigment development is markedly influenced as to degree and color by factors such as *p*H, the presence of amino acids, the presence of reducing agents such as ascorbic acid, etc. Nevertheless, the development of color upon the addition of aqueous solutions of phenols to various plant tissues is still used as a qualitative means of detecting phenolase action (26). It should be pointed out that this test is not always reliable, for if any significant amount of reducing material such as ascorbic acid is also present in the plant tissue, there is likely to be no color development for some time, even though the tissue contains appreciable amounts of tyrosinase or other phenolases.

1. Monophenolase (Cresolase) Activity

Graubard and Nelson (35) while studying the action of crude tyrosinase preparations from the potato on *p*-cresol and catechol observed that, when relatively small amounts of the enzyme were used, the rate of oxidation of *p*-cresol, after the initial lag period in the reaction, was directly proportional to the enzyme concentration. They followed the oxidation by oxygen-uptake measurements using a Warburg respirometer at 25° C., and proposed that the amount of enzyme causing an oxygen uptake of 10 mm.^{3} per minute be defined as 1 unit. This definition of a unit of tyrosinase activity (10 mm.^{3} O$_2$ uptake per minute) has been used in all subsequent work on tyrosinase in our laboratory. However, the conditions of the measurement have been altered slightly from time to time as the result of experience with more and more highly purified enzymes.

Adams and Nelson (2) found, for example, that when purified preparations of tyrosinase are highly diluted for activity measurement they rapidly lose activity. If an inert protein such as gelatin, egg albumin, serum albumin, casein, etc., is added during the dilution or immediately thereafter, this loss in activity is prevented and the activity measurements run proportional to the dilution factor. Furthermore, they observed that inert protein in the reaction system markedly cut down inactivation of the enzyme occurring during the oxidation of *p*-cresol, that is, the reaction course was linear for a longer period of time. For these reasons they proposed that 5 mg. of gelatin be present in the *p*-cresol enzyme system during the activity measurement.

More recently, Miller and Dawson (52) reinvestigated the use of gelatin in the determination of the cresolase activity of tyrosinase. They found that, whereas gelatin al-

ways gave a boost in activity, the extent of the increase in activity was dependent on the type of tyrosinase employed. The cresolase activity of tyrosinase preparations relatively high in monophenolase action was increased much more by the use of gelatin in the determination than was the cresolase activity of high catecholase preparations. Furthermore, the effect of gelatin was found to be markedly dependent on the p-cresol concentration. They concluded that gelatin was acting in some more complex way than as a simple protective agent, possibly by modifying the enzyme–substrate relationships during the course of the reaction.

2. Catecholase Activity

A number of different methods are described in the literature for the quantitative measurement of the activity of the potato or mushroom oxidase toward catechol. The reason why several different methods have been used lies in the following. During the aerobic oxidation of catechol as catalyzed by the enzyme, it is usually observed that the rate of oxygen consumption falls off rapidly from the moment the reaction is initiated and, unless relatively large amounts of the enzyme are employed initially, the oxidation stops before the catechol is completely oxidized. In other words, the oxygen-uptake curve with time is linear only for a very short time, if at all, due to rapid inactivation of the enzyme during the reaction (see Fig. 1). It is therefore difficult to obtain reliable measurements of the initial rate of oxidation by this method, particularly if purified enzyme is employed, for generally the inactivation occurring during the reaction becomes more pronounced with each successive stage in the purification. Different investigators have attempted to get around this problem of enzyme inactivation in different ways.

Graubard and Nelson (35) used the volume of oxygen absorbed in 60 minutes (see Fig. 1) as a measure of the catecholase activity of the enzyme rather than the initial rate of oxidation. Subsequent experience in the authors' laboratory has shown, however, that all tyrosinase preparations, even from the same source, are not inactivated in the same manner (see Section V). Some are inactivated very rapidly during the catechol reaction, and others much more slowly. Thus, a unit of catecholase activity based on the volume of oxygen absorbed during the inactivation process varies in magnitude from one preparation of the enzyme to another, depending largely on the method of isolation and purification employed.

Keilin and Mann (44) and Gregg and Nelson (36), using Barcroft differential manometers (31), increased the rate of shaking and took readings of oxygen uptake every minute for the first 2 or 3 minutes of the reaction in order to estimate the initial reaction velocity. Keilin and Mann employed a 3.3-ml. reaction volume containing enzyme and 5 to 10 mg. of catechol in dilute phosphate buffer (pH 7.3). They made the measurements at 20° C. while shaking the manometers at about 180 oscillations per minute. The activity of the enzyme toward catechol was expressed as Q_{O_2} calculated from the velocity during the first 2 minutes of the reaction. Gregg and Nelson used an 8.0-ml.

reaction volume containing, in addition to enzyme and 4 mg. of catechol, dilute phosphate–citrate buffer (pH of system, 7.1) and 5 mg. of gelatin.   The use of gelatin as an agent to "protect" the enzyme against inactivation during the reaction had been suggested earlier (2).   Measurements were made at 25° while shaking the manometers at about 160 oscillations per minute.   One unit of catecholase activity was defined as that amount of enzyme which would bring about an oxygen uptake of 10 mm.[3] per minute, an average minute interval value (usually the first or second) from several manometers being used as the basis for the calculation.   Parkinson and Nelson (63) estimated that
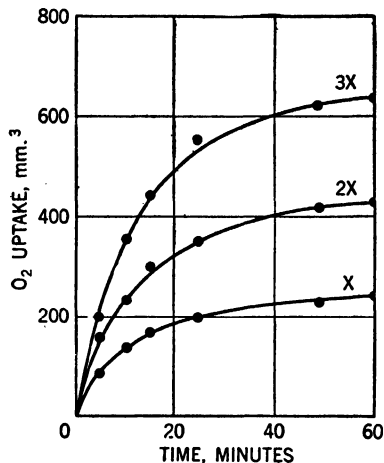
Fig. 1.—The oxidation of 4 mg. catechol at pH 6.2 and 25° C. by three different amounts (x, 2x, 3x) of partially purified mushroom tyrosinase (Graubard and Nelson, 35). The complete oxidation of 4 mg. catechol to the two-oxygen atom stage corresponds to an uptake of 832 mm.[3]

catecholase activity measurements made by this method were reliable to ±20–30%. Keilin and Mann made no statements regarding the possible error in their measurements, but it is to be noted that they used very large amounts of the enzyme for the determinations, recording oxygen uptakes as high as 78 mm.[3] per minute.   Experience in the authors' laboratory, where it has been observed that an oxygen uptake of 30–40 mm.[3] per minute is about the uppermost safe working range even when the rate of shaking of the manometers is 150–160 oscillations per minute, would lead one to wonder how much the rate values reported by Keilin and Mann were influenced by the rate of shaking, i. e., by the rate of diffusion of air into the reaction system.

Keilin and Mann also used a colorimetric method based on estimating the amount of purpurogallin formed from pyrogallol at 20° C., and defined one unit of the enzyme as

that amount which would produce 1000 mg. of purpurogallin in 5 minutes. It is difficult to compare this method and its unit with other methods employing catechol as the substrate, for it has been observed (75) that small variations in the structure of the catechol molecule not only may greatly change the rate of oxidation affected by a given amount of enzyme, but may also change the degree of enzyme inactivation occurring during the reaction. It has been pointed out previously that the reaction stability of the enzyme, i. e., its degree of inactivation occurring during the initial stages of the oxidation of catechol, varies from one enzyme preparation to another. It follows that a unit of activity based on a single rate measurement made at the expiration of 5 minutes fails to take this stability factor into account.

Employing purified high catecholase and high cresolase preparations of tyrosinase, Miller and Dawson (50, 51) studied in some detail the direct manometric method as used by Gregg and Nelson for measuring the catecholase activity of the enzyme, and made some pertinent observations regarding the reliability of the method and the "protective" role of gelatin in the reaction system. It was found necessary to use the average of many rate determinations in order to obtain a reliable activity value based on dO_2/dt within the first minute or two of the reaction. The "protective" action of gelatin was found to be variable and to depend on a number of factors, including the nature of the enzyme. No significant difference in the maximum rate of oxidation was observed between pH 5.5 and 7.1. As the result of these studies the need for a reliable method of measuring the initial reaction velocity of the enzymatic oxidation of catechol in the absence of "protective" protein became apparent.

Because of the relatively high oxidation–reduction potential of the o-benzoquinone–catechol system ($E_0 = 0.792$, ref. 10) it is possible to oxidize aerobically, by means of tyrosinase and a catalytic trace of catechol, a variety of substances (reductants) upon which the enzyme has no direct action. The small amount of catechol is rapidly oxidized by the enzyme to o-benzoquinone, and the latter is continuously reduced back to catechol by the reductant. The net result is an oxidation of the reductant with the consumption of oxygen. At no time during the oxidation of the reductant does any appreciable quantity of o-benzoquinone exist in the system.

Kubowitz (45) made use of the above principle for testing the catecholase activity of tyrosinase. He believed that the marked inactivation of the enzyme during the direct oxidation of catechol was due to a destructive action of o-benzoquinone on the enzyme protein, and therefore reasoned that, if the quinone concentration were always very low during the reaction, there would be little or no inactivation. He estimated the activity of his potato oxidase preparations by determining the rate of aerobic oxidation of hexose monophosphate in the presence of its dehydrogenase, coenzyme II, and a very small amount (0.02 mg.) of catechol. The essential reactions of his system are:

$$\text{Hexose monophosphate} + \text{coenzyme II} \xrightarrow{\textit{dehydrogenase}}$$

$$\text{phosphohexonate} + \text{reduced coenzyme II} \quad (7)$$

$$\text{Reduced coenzyme II} + o\text{-quinone} \longrightarrow \text{coenzyme II} + \text{catechol} \quad (8)$$

$$\text{Catechol} + \text{oxygen} \xrightarrow{\textit{oxidase}} o\text{-quinone} + \text{water} \quad (9)$$
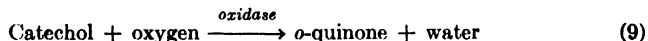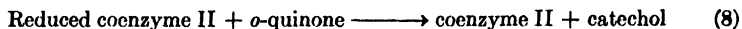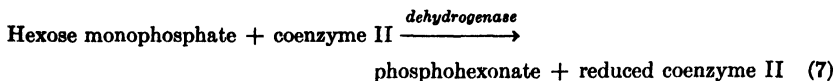
Using the amount of oxygen absorbed by the system during the first 10 minutes, he expressed the activity (w) of the enzyme as:

$$w = \frac{\text{mm.}^3\ O_2}{\text{mg. enzyme} \times \text{minutes}} \quad (10)$$

Kubowitz presented data showing that the rate of oxygen uptake was proportional to the enzyme, and that the reaction course under these conditions was approximately linear for over 20 minutes. It should be pointed out, however, that the data presented were for crude potato extracts and experience shows that the inactivation problem is much less serious for crude preparations of the enzyme, particularly from the potato. It should also be mentioned here that it has since been established that o-benzoquinone is not responsible for the inactivation of the enzyme during the oxidation of catechol. When purified enzyme preparations are employed for oxidizing catechol, the degree of inactivation of the enzyme during the reaction is not changed when the reductant, ascorbic acid, is made an additional component of the system (see Section V).

Kubowitz stated that the activity measurement on the enzyme could be increased by increasing the concentration of catechol in the system, the rate of oxidation being proportional to both the enzyme and catechol concentrations under the conditions employed. Since he did not state the activity of his preparations using an optimum concentration of catechol, which in the experience of the authors would have produced a much higher activity value, it is difficult to compare the activity of his purified enzyme preparation with those that have been measured using a much higher concentration of catechol in the reaction system. Direct comparisons on the basis of Q_{O_2} have little meaning. Failure to recognize this fact has led to delusive statements in the literature regarding the relative catecholase activities of potato and mushroom tyrosinase.

Kubowitz found that, for a given amount of the enzyme, the greatest rate of oxidation of catechol occurred at pH 7.1, and reported that the rate was greatly reduced (about one-half) when systems buffered to pH 6.1 and 8.0 were used. Such a marked dependency of the rate of oxidation on the pH of the catechol–enzyme system has never been observed by the authors using either mushroom or potato tyrosinase. In fact, as previously mentioned, no significant difference was found between the rate at pH 7.1 and that at pH 5.5 using mushroom tyrosinase (50). In view of this fact, it would seem likely that the rates of oxidation as measured by Kubowitz at pH 6.1 and 8.0 were limited by other factors in the system than the potato oxidase. Probably because of the complexity of the system and the fact that it involves agents that are not always easily available, Kubowitz' method of measuring catecholase activity has not found wide application.

The following year, Adams and Nelson (3) suggested that a simpler and much more easily available reductant, hydroquinone, could be used in the catechol–enzyme system for the purpose of maintaining a low con-

centration of *o*-benzoquinone during the activity measurement. Tyrosinase has no direct action on hydroquinone, but, on the addition of a catalytic amount of catechol, rapid oxidation ensues. The enzyme seems to suffer less inactivation when the catechol–hydroquinone system is used; and when gelatin is also a component of the system the reaction course as followed manometrically is frequently found to be linear for over one-half hour. Adams and Nelson used an 8.0-ml. reaction volume at 25° C. containing, in addition to enzyme, 5 mg. of hydroquinone, 0.1 mg. catechol, dilute phosphate–citrate buffer (*p*H of system, 7.0), and 5 mg. of gelatin. Measurements of oxygen uptake were made at 5-minute intervals while shaking the manometers at about 90 oscillations per minute. One catecholase unit was defined as the amount of enzyme required to cause the rate of oxidation to equal 10 mm.[3] per minute.

Because of its simplicity and the fact that the linear reaction course made reproducible rate measurements of the enzymatic oxidation of catechol easily obtainable for the first time, the catechol–hydroquinone–gelatin method met with favor. It did much to stimulate quantitative comparison studies of the catecholase activity and monophenolase activity of tyrosinase, and in this sense played an important role in the development of our present knowledge concerning the mode of action of tyrosinase. It is now known, however, that the catechol–hydroquinone–gelatin method does not give a true measure of the catecholase content of tyrosinase. The continued use of the method, therefore, is open to serious criticism. For example, more recent studies have shown that hydroquinone does not act merely as a reductant for the *o*-benzoquinone: under certain conditions it is oxidized by the enzyme (37); and in all cases it acts as an inhibitor in the system. The initial rate of enzymatic oxidation of the catechol–hydroquinone mixture is always much lower than the rate observed with catechol alone, *i. e.*, catecholase activity obtained by the catechol–hydroquinone method is always less than that obtained using the direct manometric method on catechol (see discussions on pages 108 and 109). As mentioned above, hydroquinone tends to stabilize the enzyme against inactivation during the reaction. If these effects of hydroquinone were constant, there would be less serious criticism to the continued use of the method; but, as a matter of fact, both the stabilizing effect and the degree of inhibition vary widely with factors such as the purity of the enzyme and the ratio of the catecholase to the cresolase activity of the enzyme (50, 63).

As mentioned earlier in the introductory part of this section on catecholase activity, tyrosinase preparations show marked differences in the

degree of enzyme inactivation which occurs during the early stages of the enzymatic oxidation of catechol. This of course means that catecholase activities based on rate measurements made after the reaction has progressed for a short time are influenced by the degree of enzyme inactivation that has occurred up to that time. Only in the case in which no inactivation occurs during the early stages of the oxidation will such a rate measurement give a true indication of the catecholase content of the enzyme. It is apparent, therefore, that, to make reliable comparisons of different tyrosinase preparations on the basis of their catecholase activities, the activity measurements should be based on the initial reaction velocity, that is, dO_2/dt at $t \rightleftharpoons 0$.

As pointed out previously, the lack of precision inherent in the manometric method, when attempts are made to take readings of oxygen uptake within the first two or three minutes of the catechol reaction, makes the determination of dO_2/dt at $t \rightleftharpoons 0$ impracticable. It is possible, however, to follow with good precision the production of o-benzoquinone during the very early stage of the reaction, $i.\ e.$, within the first 20–120 seconds. The method developed in the authors' laboratory for this purpose (53) has been called the chronometric method, since it involves a measurement of the time required for a given quantity of enzyme to produce o-benzoquinone just in excess of that necessary to oxidize a small amount of ascorbic acid also present in the system. During the enzymatic oxidation of a mixture of catechol and ascorbic acid* no detectable amount of o-benzoquinone exists in the solution, and the reaction mixture remains perfectly colorless until the moment the ascorbic acid (AH_2) has all been converted to dehydroascorbic acid (A):

$$
\text{(11)}
$$

$$
\text{(12)}
$$
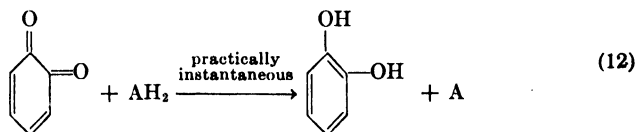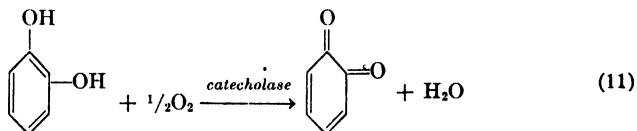
The end point time (time of appearance of excess o-benzoquinone in the system) is indicated by a blue coloration at the point at which the reaction mixture is continuously

---

* The ascorbic acid functions only as a reductant and does not inhibit or accelerate the enzyme action. It is not directly oxidized by the enzyme nor does it affect the inactivation characteristics of the enzyme (46, 51).

sampled dropwise (by means of a capillary tube acting as a syphon) into an acidified starch–iodide indicator solution. The acid in the indicator solution stops the enzymatic reaction, and no coloration develops with each successive drop until the moment the ascorbic acid has been completely oxidized in the reaction system. The next drop, containing *o*-benzoquinone, liberates iodine in the indicator and a blue coloration appears simultaneously in the immediate vicinity of the drop. If the experiment is set up a number of times using different amounts of ascorbic acid with a given amount of enzyme and optimum amount of catechol, a series of end-point times is obtained. In this way the production of *o*-benzoquinone (or its equivalent the disappearance of ascorbic acid) can be measured as a function of time for a given amount of the enzyme.

The method is nothing more, therefore, than an indirect titration of *o*-benzoquinone, performed in such a manner that the quinone is never present in the reaction mixture until the instant of detection. This eliminates from consideration the many complex side reactions which make the direct titration of *o*-benzoquinone unsuitable for measuring the enzyme activity (29). By using small amounts of ascorbic acid in the system, the *o*-benzoquinone production can be followed with good precision during the very early stage of the reaction. The amount of catechol used is dependent on the nature of the tyrosinase preparation, for the optimum substrate concentration, *i. e.*, the concentration of catechol yielding a maximum activity of the enzyme, varies with the purity of the enzyme and the ratio of the catecholase to cresolase activities. In general it is found that crude tyrosinase preparations and preparations that are relatively high in monophenolase action (high cresolase preparations) have a much higher optimum catechol concentration than do high catecholase preparations of tyrosinase. In all cases the amount of catechol used is much greater (mole basis) than the amount of ascorbic acid.

A study of the initial reaction course by means of this chronometric technique has revealed (53) that, during the first two or three minutes of the oxidation of catechol as catalyzed by tyrosinase, *o*-benzoquinone is produced in accordance with the following expression:

$$Q = \frac{at}{b + t} \tag{13}$$

where Q is *o*-benzoquinone, a and b are constants determined by the degree or extent of enzyme inactivation occurring during the initial stage of the reaction, and t is the time of the reaction in seconds.

When the values of a and b are relatively small in comparison with t, the equation expresses the fact that the maximum rate of oxidation (maximum rate of quinone production) is the initial rate ($t \rightleftharpoons 0$), and that the rate then falls off rapidly as the reaction progresses, that is, as t increases. It will be recalled that this is the case when purified high catecholase preparations of tyrosinase are employed, for the enzyme is rapidly inactivated during the early course of the reaction (see Fig. 2).

The values of a and b, which determine the curvature of the reaction course, may be evaluated by determining the slope and intercept of the linear curves obtained when $1/Q$ is plotted against $1/t$ as expressed by the reciprocal equation:

$$\frac{1}{Q} = \left(\frac{b}{a}\right)\left(\frac{1}{t}\right) + \frac{1}{a} \tag{14}$$
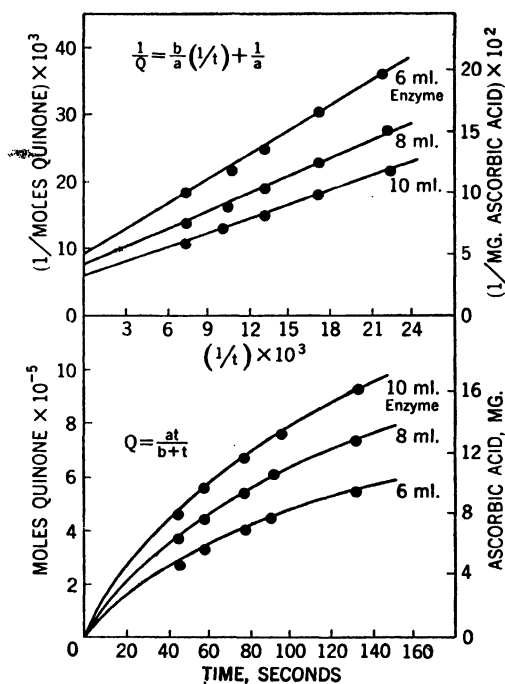
Fig. 2.—The production of o-benzoquinone during the initial stage of the enzymatic oxidation of 50 mg. catechol (250 ml. reaction volume) at pH 5.5 and 25° C. Aerobic oxidation catalyzed by three different amounts (6, 8, and 10 ml.) of a diluted solution of purified high-catecholase preparation of mushroom tyrosinase (Miller, Mallette, Roth, and Dawson, 53).

Differentiation of Eq. 13 in respect to t gives:

$$\frac{dQ}{dt} = \frac{ab}{(b+t)^2} \tag{15}$$

and it follows that, when $t \rightleftarrows 0$:

$$\frac{dQ}{dt}_{(t \rightarrow 0)} = \frac{a}{b} = \text{initial reaction velocity} \tag{16}$$

Thus, the initial reaction velocity (a/b) is the reciprocal of the slope of the linear curves obtained by plotting $1/Q$ vs. $1/t$ (see Fig. 2), and may easily be determined from the experimental data. Maintaining the definition of a unit of enzyme as that amount which will cause an oxygen uptake of 10 mm.[3] per minute, it follows that the initial reaction velocity (a/b), which is obtained in moles of o-benzoquinone formed per second, can be converted into catecholase units by:

$$\text{Catecholase units} = (a/b)/1.49 \times 10^{-8} \qquad (17)$$

since an oxygen uptake of 10 mm.[3] per minute (1 unit) is equivalent to an o-benzoquinone production of 1.49×10^{-8} moles per second. (If Eqs. 13 or 14 are expressed in terms of mg. of ascorbic acid rather than moles of quinone, then catecholase units $= (a/b)/2.62 \times 10^{-3}$, since 2.62×10^{-3} mg. of AH_2 is equivalent to 1.49×10^{-8} moles of AH_2 or o-benzoquinone.)

If no inactivation of the enzyme is apparent during the first two or three minutes of the reaction, i. e., the production of o-benzoquinone with time is linear, then the values of a and b are very large in respect to t (a and b approach infinity) and Eq. 13 reduces to the expression for a straight line through the origin:

$$Q = at/b = kt \qquad (18)$$

This is the case often observed when crude extract preparations or purified high cresolase preparations of tyrosinase are employed in the oxidation of catechol. The initial reaction velocity ($a/b = k$) is obtainable directly from the original data, and it is not necessary, therefore, to plot $1/Q$ vs. $1/t$ in order to calculate the catecholase activity of the enzyme (Eq. 17).

Catecholase activity values based on the initial reaction velocity, as determined by the chronometric method, are proportional, within certain limits of enzyme concentration, to the amount of enzyme introduced into the system. They are always considerably higher, however, than those obtained by the direct manometric method on catechol previously described (36). The reason is apparent when one is dealing with an enzyme which is rapidly inactivated during the initial stage of the reaction, for the rate measurement in the manometric method is made after the reaction has progressed for a while, and is therefore influenced by the degree of enzyme inactivation prior to the measurement. In the case in which no inactivation of the enzyme is noticeable during this early stage of the reaction, the higher value obtained by the chronometric method appears to be due largely to the fact that no gelatin is employed in this method, and the diluted enzyme is not subjected to a long temperature equilibration period prior to the measurement. Both these factors have been shown to lower the catecholase activity of the enzyme when the activity is based on the first-minute reading of oxygen uptake in the manometric method (50)

V.   The Stability and Inactivation of Tyrosinase

It is well recognized that enzyme solutions vary greatly in stability, and that many factors, such as enzyme concentration, enzyme purity, pH, temperature, microorganisms, foreign ions, etc., greatly influence the stability of enzyme solutions. Some enzymes, such as catalase and peroxidase, can stand relatively rough treatment without appreciable loss in activity. In other words, these enzyme proteins are relatively stable to denaturation. Other enzyme solutions, however, lose activity quite rapidly even when the solution is adjusted to optimum pH, the temperature is kept low, and microorganisms are excluded by the use of an antiseptic such as toluene. The protein moieties of such enzymes are very fragile and are easily denatured, possibly spontaneously. Sometimes enzymes are more stable in the dry form, but frequently the stability properties of the dried enzyme depend on the degree of purification affected before the drying operation. Likewise, the manner in which the water is removed generally has great influence.

The most pronounced stability properties of crude tyrosinase have already been described (Section II). As a generalization, it can be stated that the stability of tyrosinase increases with purification, but it must be kept in mind that the concentration of the enzyme solution is important in this respect. Purified tyrosinase solutions, that contain in excess of about 1 mg. of enzyme protein per ml. of solution, generally show little loss in activity over a period of several months if they are buffered to about pH 7, inoculated with a few drops of toluene, and stored at refrigerator temperatures. The same enzyme solution, however, when highly diluted for activity measurements, may exhibit a significant loss in activity in 15 or 20 minutes even though the temperature of the diluted enzyme is maintained at 0–5° C. The process, which is probably a dissociation or unfolding of the enzyme protein into less active forms, is considerably more rapid at room temperature. Inert protein material, such as gelatin, impedes the process (2), and apparently can also reverse it if it has not gone too far (50).

The monophenolase (cresolase) activity of the enzyme is less stable than the catecholase activity, as a general rule. This is in line with the observation that high cresolase preparations of tyrosinase are much more difficult to prepare and preserve than high catecholase preparations. Losses in total activity which occur during the preparation process, due probably to protein denaturation, generally result in an increase in the ratio of catecholase to cresolase activity in the remaining active enzyme.

Conditions known to result in protein denaturation, such as the application of heat (60° C.) for a short period, or vigorous shaking of the protein solution in air for several hours, have been reported not only seriously to inactivate the enzyme but also to cause a significant increase in the ratio of catecholase to cresolase activity (3, 83). In this connection it should be emphasized that, to the authors' knowledge, enzyme preparations possessing only monophenolase activity have never been obtained from the common mushroom or potato. The monophenolase activity appears to be dependent on, and closely associated with, the catecholase activity of the enzyme (see Section VI).

As previously pointed out (see Section II), tyrosinase is made inactive by any agent which combines irreversibly with copper, or any condition that removes copper from the enzyme protein, such as dialysis at low pH. Gregg and Nelson (36) found that the cresolase and catecholase activities of tyrosinase were affected to the same extent by cyanide and diethyldithiocarbamate, irrespective of the original ratio of the activities of the enzyme

1. Inactivation during the Enzymatic Oxidation of Catechol

One of the most striking characteristics of the enzymatic oxidation of catechol, as catalyzed by purified high catecholase preparations of tyrosinase, is the pronounced inactivation or destruction of the enzyme that occurs during the course of the reaction. Some aspects of the phenomenon have already been described in connection with the discussion of the measurement of catecholase activity (Section IV). The enzyme inactivation is so readily apparent during the course of the reaction that many investigators have concerned themselves with the problem at one time or another. Because of the character of the inactivation, it seemed logical at first to attribute it to the destructive action of products formed during the course of the oxidation. Hydrogen peroxide and o-benzoquinone have frequently been mentioned as the inactivators (18, 35, 45, 72). However, hydrogen peroxide is not formed in detectable amounts in the tyrosinase–catechol system (Section III), and Ludwig and Nelson (46) conclusively demonstrated that the inactivation is not due to o-benzoquinone or any of its polymerization products. The loss in catecholase activity of the enzyme that occurs during the reaction is irreversible, and tyrosinase thus inactivated is also inactive toward monophenols (36).

Ludwig and Nelson were the first to attempt a quantitative examination of the enzyme inactivation occurring during the oxidation of catechol. Using purified high catecholase preparations of tyrosinase, they demon-

strated that the amount of oxygen absorbed before the enzyme became completely inactivated during the oxidation was directly proportional to the amount of enzyme employed. The inactivation at 25° C. was found to involve an oxygen uptake of 100 ± 5 mm.[3] per unit of enzyme, or 3.3×10^4 mm.[3] per microgram of enzyme copper. (The catechol–hydroquinone–gelatin unit of Adams and Nelson was used. See Section IV.) Ludwig and Nelson found that this inactivation total varied with the source of the tyrosinase and the temperature of the reaction system, but was independent of the rate of oxidation of the catechol, the concentration of oxygen in the system, the hydrogen-ion concentration between pH 5 and 7.5, and the purity of the enzyme after a certain stage in purity had been reached. Permitting the enzyme to remain in contact with o-benzoquinone for a considerable time before initiating the reaction, by adding catechol, had no effect on the inactivation total. Likewise, anaerobic incubation with catechol did not affect the inactivation characteristics of the enzyme. The enzyme was inactivated in the same manner when a mixture of ascorbic acid and a very small amount of catechol was used as the substrate. In this case, the ascorbic acid was indirectly oxidized by reducing the o-benzoquinone back to catechol as rapidly as formed, and thus polymerization products of the quinone were at no time existent in the reaction system during the inactivation. Ludwig and Nelson concluded that:

"The inactivation is not due to any products known to be formed during the oxidation of catechol, but occurs at the time the catechol is oxidized."

They suggested that the volume of oxygen absorbed during the inactivation of the enzyme could be used as a measurement of the amount of enzyme in the catechol–enzyme system. Later, however, Parkinson and Nelson (63) found that high cresolase preparations of tyrosinase did not inactivate as rapidly during the oxidation of catechol, that is, these preparations were found to give a higher inactivation total per γ of enzyme copper.

As the result of more recent studies (50), it has become apparent that the oxygen inactivation totals obtained by manometric methods are an over-all measure of the effect of a variety of factors contributing to the enzyme destruction. When the enzyme inactivation is a relatively slow process, as is the case when crude enzyme or high cresolase tyrosinase is used, and the oxidation proceeds for a half hour or more before the rate of oxygen uptake approaches zero, the oxygen inactivation totals are greatly influenced by such factors as the rate of shaking of the manometers and the concentration of buffer and inert protein material in the reaction medium. On the other hand, when the inactivation of the enzyme is

rapid, *i. e.*, the oxidation of the catechol stops within a few minutes after initiating the reaction, the inactivation totals are little affected by variation in the factors mentioned above. Such is the case when purified high catecholase preparations of tyrosinase are employed.

It has been known for a long time that many proteins tend to coagulate when their aqueous solutions are vigorously shaken. Being surface-active agents, they tend to concentrate at the air–liquid interface and to spread to form a protein film. During this spreading process they are frequently denatured (21). Tenenbaum (83) has shown that tyrosinase is inactivated by shaking, or by bubbling air or oil through aqueous solutions of the enzyme, and that the addition of other surface-active agents such as soap, gelatin, octanol, etc., protect the enzyme by preventing it from entering the interface and becoming denatured. It appears likely, therefore, that, for a case in which the inactivation of tyrosinase during the oxidation of catechol requires considerable time, *i. e.*, when crude enzyme or high cresolase preparations of tyrosinase are employed, possibly a major portion of the inactivation is due to surface denaturation of the enzyme. Presumably, a fresh air–liquid interface is formed with each oscillation of the manometer flask. Thus, the oxygen inactivation total is affected by the rate of shaking and is greatly increased (less enzyme inactivation) when gelatin is made a component of the system. Since these factors have much less effect on the oxygen inactivation totals of a purified high catecholase preparation of tyrosinase, and serious inactivation of the enzyme occurs within the first minute or two of the catechol oxidation, the major portion of the inactivation in this case must be due to some other reason than surface denaturation. Keeping in mind that all known products of the oxidation of catechol have been eliminated in this consideration, it would appear that the inactivation occurs as a direct result of the catalytic action of the enzyme and is due to some factor inherent in the catechol–enzyme–oxygen system.

2. The Theoretical Inactivation Constant

The chronometric method, as indicated in Section III, for following the enzymatic oxidation of catechol, has made possible a study of the primary inactivation of the enzyme, that is, the inactivation occurring during the first minute or two of the reaction apparently as a direct result of the catalytic function of the enzyme.

It will be recalled that, during this stage of the reaction, the production of *o*-benzoquinone (Q) with time (*t* in seconds) for a given amount of tyrosinase is in accordance with the following expressions:

$$Q = \frac{at}{b + t} \tag{13}$$

or

$$\frac{1}{Q} = \left(\frac{b}{a}\right)\left(\frac{1}{t}\right) + \frac{1}{a} \tag{14}$$

It follows from Eq. 14 that when t is infinity, $Q = a$. In other words, a, the reciprocal of the intercept obtained by plotting $1/Q$ vs. $1/t$ (see Fig. 2) is the amount of quinone that could be produced in infinite time by a given quantity of the enzyme, provided Eq. 13 were to hold throughout the entire reaction course.

The term a has therefore been called the "theoretical inactivation constant" of the enzyme (75), and is a measure of the amount of oxidation a given quantity of the enzyme can catalyze before becoming completely inactivated when the inactivation of the enzyme occurs only as the direct result of its catalytic reaction. Actually, Eq. 13 holds only during the initial stage of the reaction, i. e., during the first two to three minutes. When the reaction course is followed for longer periods of time, it gradually departs from that expressed by Eq. 13 because of surface denaturation of the enzyme. For this reason, the actual extent of oxidation before complete inactivation of the enzyme (the oxygen inactivation total) is always less than that expressed by the "theoretical inactivation constant." The value in the latter lies in the fact that it gives a measure of the inactivation due only to the catalytic function of the enzyme, and it can be compared over the same period of the reaction for different tyrosinase preparations or for different o-dihydric phenols.

As previously inferred, purified high catecholase and high cresolase preparations of tyrosinase show a marked difference in the degree of enzyme inactivation that occurs during the initial stage of the enzymatic oxidation of catechol. Experiments now in progress in the authors' laboratory indicate that the value of a may be used as a characterizing property or constant of the type of tyrosinase. For example, a considerable number of purified high catecholase preparations of tyrosinase have been found to yield very close to the same value of a, even though their purity (catecholase activity per γ of copper or per mg. dry weight) and their ratio of catecholase to cresolase activity varied over a considerable range. The value of a for purified high cresolase preparations is much higher than that for high catecholase preparations, which is another way of stating that much less inactivation of the catecholase activity of the enzyme occurs in the initial stage of the catechol oxidation when the former type of tyrosinase is employed. (Several purified high cresolase preparations have been found to yield a value of a approaching infinity, i. e., no perceptible inactivation of the catecholase activity of the enzyme occurred during the first two minutes of the catechol oxidation, and therefore the value of $1/a$ in Eq. 14 was determined as zero in the $1/Q$ vs. $1/t$ plot.)

3. The Influence of Catechol Structure

The degree of enzyme destruction that occurs during the first minute or two of the enzymatic oxidation of an *o*-dihydric phenol is not only dependent on the type of enzyme employed but is also greatly influenced by variations in the structure of the *o*-dihydric phenol. Thus, it has been found (see Table II) that, whereas a purified high catecholase preparation of tyrosinase is rapidly inactivated during the enzymatic oxidation of catechol, 4-methylcatechol, and epinine, no inactivation during the initial stage of the reaction is observed when 4,5-dimethylcatechol, 4-*tert*-butylcatechol or 3-methylcatechol are used as substrates. The data in the table also show that the initial rate of enzymatic oxidation and the optimum substrate concentration are likewise markedly influenced by the structure of the *o*-dihydric phenol. It can be seen that the degree of enzyme inactivation (indicated by the inverse value of *a*) shows no simple relationship to the rate of oxidation, to the affinity of the enzyme for the dihydric phenol, or to the structure of the catechol molecule.

Although such studies of the enzyme inactivation occurring during the initial stage of the enzymatic oxidation of catechol show promise of yielding some insight into the mode of action of the enzyme, it is not possible at present to postulate a completely satisfactory explanation or mechanism for the rapid enzyme destruction. Recalling that all known products of the enzymatic oxidation of catechol have been eliminated as possible inactivators, it would seem that the inactivation must be due to some factor peculiar to the manner in which the enzyme manipulates the substrate molecules during the oxidation process. Possibly it is a collision factor during the formation and dissociation of the enzyme–substrate complex. It might just as likely, however, be a strain or energy factor within the enzyme-substrate complex. The dependency of the phenomenon on the structure of the catechol molecule and on the nature of the enzyme lends some support to such views.

The possibility that the enzyme inactivation might be due to some highly transient intermediary "redox" form of oxygen, *i. e.*, a precursor of water or hydrogen peroxide, has been debated many times in the authors' laboratory. The postulation is an appealing one, for it can be expanded to account for the fact that the oxidation of a monophenol as catalyzed by tyrosinase requires the simultaneous oxidation by tyrosinase of an *o*-dihydric phenol (see Section VI). In other words, it would assume that the monophenol oxidation is brought about by the enzyme through the use of an intermediary "redox" or active form of oxygen produced during the

catechol oxidation. Less inactivation of the enzyme would therefore be expected during the monophenol oxidation. Although it is an experimental fact that the inactivation of the enzyme is much less serious during the oxidation of a monophenol, the data in Table II, showing that the inactivation of the enzyme during the oxidation of an *o*-dihydric phenol bears no relationship to the rate of oxidation, make such an explanation of the enzyme inactivation appear very unlikely. If one assumes that the

TABLE II

SOME FACTORS INFLUENCING DEGREE OF ENZYME INACTIVATION*

Substrate	Catechol	4-Methyl-catechol	4,5-Dimethyl-catechol	Epinine* hydro-chloride	4-*tert*-Butyl-catechol	3-Methyl-catechol
Optimum concentration, mg./100 ml.	20	7.5	10	100	100	150
Initial rate (a/b), (moles Q/sec./γ enzyme) $\times 10^8$	1.933	1.713	1.439	1.258	0.901	0.850
Theoret. inact. value (a) (moles Q/γ enzyme) $\times 10^6$	2.89	2.55	$a \longrightarrow \infty$ lim.	3.77	$a \longrightarrow \infty$ lim.	$a \longrightarrow \infty$ lim.
Per cent of catechol rate per γ enzyme	100.0	88.5	74.4	65.4	46.9	4.4

* Showing how the optimum concentration of substrate, initial rate of oxidation, and theoretical inactivation value (a) vary with different *o*-dihydric phenols using a purified high catecholase preparation of tyrosinase (Roth, Miller, and Dawson, 75). Epinine is 4-(*N*-methyl-2'-aminoethyl)-catechol.

enzymatic oxidation of catechol to *o*-benzoquinone produces some transient "redox" form of oxygen, one may also safely assume that the same intermediate will be involved in the enzymatic oxidation of various substituted catechols. Furthermore, the amount of oxygen intermediate formed in any given time should bear a definite relationship to the number of molecules of the catechol oxidized. It follows, therefore, that if such an active form of oxygen were responsible for the inactivation of the enzyme, one would expect the inactivation to bear a definite relationship to the amount of oxygen absorbed irrespective of the structure of the catechol being oxidized. It is obvious that the same argument can be used against hydrogen peroxide's being the inactivator.

VI. The Enzymatic Oxidation of Monohydric Phenols

On turning to the oxidation of the monohydric phenols by means of tyrosinase, it soon becomes apparent that the situation is more complicated than in the case of the o-dihydric phenols. This is because three atoms of oxygen instead of two, as in the case of the o-dihydric phenols, are consumed per molecule of the monohydric phenol oxidized, and the reaction course as followed by oxygen-uptake measurements is characterized by an initial lag or induction period (see Fig. 3). In other words, the reaction is complicated by being autocatalytic in character.

All the available evidence supports the early view (70) that the first stage in the oxidation is a conversion of the monophenol into its corresponding catechol compound, and that the catechol is then oxidized to an hydroxyquinone with the consumption of two atoms of oxygen (see Section III). Thus, the additional atom of oxygen uptake, observed in the monophenol oxidation, is used to convert the monophenol into the o-dihydric form. The question which has proved very difficult to answer, and around which most of the controversy over tyrosinase action has developed, has been whether the conversion of the monophenol into its catechol compound is a function of the enzyme or is brought about by oxidation products resulting from the enzymatic oxidation of the o-dihydric phenol. The autocatalytic nature of the reaction course made the latter view appear most probable to the earlier workers, particularly after Onslow and Robinson (61) had observed that the addition of catechol accelerated the oxidation of p-cresol by tyrosinase. As pointed out in the introduction, these investigators concluded that the p-cresol was oxidized spontaneously by the o-quinone or possibly hydrogen peroxide formed during the enzymatic oxidation of the catechol. Recently, Califano and Kertész (22), finding the addition of catechol removed the induction period when tyrosine was oxidized by means of the enzyme, concluded, as did Onslow and Robinson, that the o-quinone was responsible for the first step in the oxidation of the tyrosine:

"The oxidation of monophenols into the corresponding o-diphenols is a simple chemical process occurring automatically.... The presumption of a tyrosinase (monophenolase) is not necessary to explain the tyrosine (monophenol) oxidation."

Instead of the monohydric phenols' being oxidized spontaneously by the o-quinones, Richter (72) has suggested that possibly tyrosinase preparations contain a monophenol dehydrogenase which enables the o-quinone to serve as a hydrogen acceptor in the oxidation of monohydric phenols.

Experiences in the authors' laboratory fail to support the above views.

It was pointed out in Section III that no satisfactory evidence for the formation of hydrogen peroxide during the enzymatic oxidation of catechol has been observed. In fact, all the evidence indicates that hydrogen peroxide is not involved in the enzymatic oxidation of monohydric phenols. Bordner and Nelson (17) added tyrosinase to a solution of p-cresol and hydrogen peroxide in the presence of an atmosphere of nitrogen, and followed the rate of disappearance of the hydrogen peroxide. They found the rate, which was very slow, was the same as that of the control experiments in which neither p-cresol nor p-cresol and enzyme were present. Turning to the possibility that o-quinones are responsible for the oxidation of the monophenols, Pugh (66) was among the first to oppose this view. She found p-cresol to be unaffected when present in a solution in which catechol was oxidized to the o-quinone by peroxidase and hydrogen peroxide. Unfortunately, her claim is weakened by the incompatibility of o-quinone and hydrogen peroxide under these conditions, as shown by Dawson and Ludwig (28). To overcome this objection, Bordner and Nelson oxidized catechol to o-quinone in the presence of phenol, using a catecholase from the sweet potato, *Ipomoea batatas*, instead of peroxidase. This catecholase has practically no action on phenol. In this case, the o-quinone formed in the oxidation of the catechol had no action on the phenol, thereby confirming Pugh's contention.

Another argument against o-quinones' being responsible for the hydroxylation of monohydric phenols is the oxidation of the latter by means of tyrosinase in the presence of benzenesulfinic acid. The sulfinic acid combines with o-quinone (41) and removes the latter quantitatively (17, 68) from the reaction mixture without preventing the enzymatic oxidation of the monohydric phenol to its o-dihydric form.

Returning to Richter's suggestion that tyrosinase preparations may contain monophenol dehydrogenases, Bordner and Nelson (17) have shown this to be improbable. They oxidized, in an atmosphere of nitrogen, some homocatechol to homoquinone by the calculated amount of ceric sulfate, and immediately after the addition of the ceric sulfate added p-cresol and tyrosinase to the reaction mixture. They then followed the rate of disappearance of the quinone. Controls were also carried out to determine the rate of disappearance of the quinone in the absence of p-cresol and enzyme as well as in the presence of the enzyme only. The results obtained showed that the rate of disappearance of the quinone was not influenced in any way by the presence of the enzyme nor by the presence of the enzyme together with p-cresol in the reaction mixture.

In the light of the evidence just reviewed, it seems safe to state that

o-quinones are not responsible for the conversion of the monohydric phenols into their *o*-dihydric forms. Yet the oxidation of *o*-dihydric phenols appears to be essential for the removal of the induction period and for enabling the enzyme to oxidize the monohydric phenol (17). Furthermore, for the oxidation of the *o*-dihydric phenol to be effective in removing the induction period, it must be caused by tyrosinase and not, as Bordner and Nelson (17) have shown, by oxidizing the *o*-dihydric phenol by other oxidizing agents, such as potassium ferricyanide or the polyphenol oxidase, laccase. The addition of such oxidizing agents to the monophenol–enzyme reaction mixture lengthens the induction period instead of removing it. This lengthening of the initial lag period by these oxidizing agents is probably due to the fact that the latter compete with the tyrosinase for the *o*-dihydric phenol as it is formed in the first stage in the oxidation. They thus eliminate the oxidation of the catechol by the tyrosinase which is necessary for the monophenolase action.

Undoubtedly, the strongest argument against the view that products of the catechol oxidation are alone responsible for the initial oxidation of the monohydric phenols is the fact that the ratio of catecholase activity to monophenol action of the enzyme can be widely varied during the preparation of tyrosinase (Section II). If the products of the catechol oxidation were alone responsible for the initial oxidation of the monohydric phenols, one should expect the two activities always to run parallel. Therefore, it is apparent that the enzyme plays a role in monohydric phenol oxidation, and the extent of this role is governed by some factor inherent in the enzyme, the amount of which in turn is governed by the particular method by which the enzyme is prepared. The most reasonable explanation concerning the monophenol activity of tyrosinase appears therefore, to be that, in the oxidation of an *o*-dihydric phenol, a portion of the enzyme complex becomes momentarily activated or primed so that it can bring about the oxidation of monohydric phenols.

1. The Induction Period

For the sake of simplicity in discussing the kinetics of monohydric phenol oxidation by means of tyrosinase, phenol and catechol will be used as examples of the two types of phenols. On the basis of the view expressed above, the enzyme is active toward phenol only when it is simultaneously catalyzing the oxidation of catechol. The following question therefore arises immediately: How can the enzyme initiate the oxidation of phenol if no catechol is added to the system? The most probable answer seems to be that, due to autoxidation of the phenol, either pre-

viously or at the beginning of the reaction, a few molecules of catechol are present in the reaction mixture. As the enzyme causes the oxidation of these few molecules of catechol, the phenolase part of the enzyme becomes activated and the oxidation of the phenol sets in. The fact that the latter reaction starts with an induction period indicates that the oxidation of the first few molecules of catechol is insufficient to activate all of the phenolase part of the enzyme combined with phenol as an enzyme–phenol complex. The rate of enzymatic oxidation of phenol seems to depend on two factors: first, the amount of enzyme in the enzyme–phenol complex; and second, the amount of activation of the complex brought about by the oxidation of catechol. Since the latter is dependent on the amount of catechol being simultaneously oxidized by the catecholase part of the enzyme, it follows that the induction period can be overcome either by the addition of catechol, or by forming and oxidizing, at the same moment, more molecules of catechol than molecules of phenol oxidized.

Of the three atoms of oxygen consumed in the oxidation of one molecule of phenol to its final oxidation product, hydroxyquinone, one atom converts the phenol into catechol and the other two oxidize two molecules of catechol (Section III) formed after the one molecule of phenol has been oxidized.
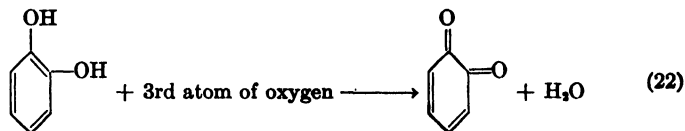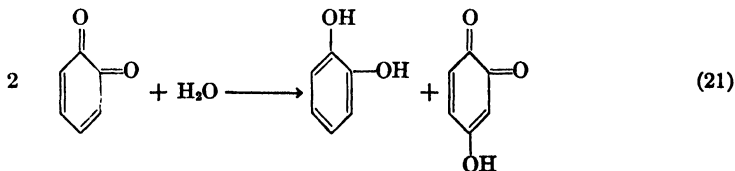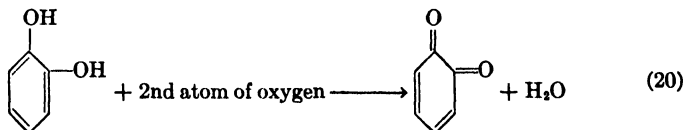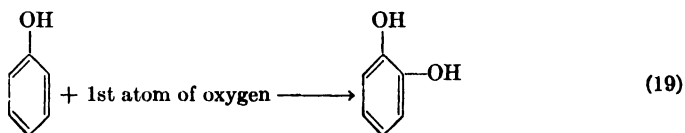
This oxidation of two molecules of catechol for every phenol molecule oxidized, coupled with the fact that the enzyme is activated toward monophenols during the catechol oxidation, seems, at present, to be the most reasonable way of accounting for the gradual autocatalytic increase in the rate of oxidation during the induction period.

Keeping the above mechanism in mind, and the fact that the enzyme is active toward monophenol only when simultaneously oxidizing catechol, it can be seen that the addition to the system of any agent which would rapidly oxidize catechol and thereby deprive the enzyme of the opportunity to oxidize catechol should lengthen the induction period. Likewise, any agent which would tend to retard the formation of the second molecule of catechol (Eq. 21) should also show a similar effect on the lag period. Any agent which would tend to reduce the quinone back to catechol should, on the other hand, shorten the induction period. The above interpretation is in keeping with experimental observations made in the authors' laboratory. For example, Bordner and Nelson (17) found that the addition of oxidizing agents such as potassium ferricyanide to the enzyme–phenol system lengthened the induction period. They also observed that the length of the induction was a function of the pH of the reaction mixture, becoming longer as the pH was lowered. This is in agreement with the observation made by Dawson and Nelson (29) that the rate of disappearance of o-benzoquinone in aqueous solutions (Eq. 21) decreases with a decrease in pH. It has likewise been noted (11, 17) that reducing agents, such as ascorbic acid, in reducing the o-quinone as it is formed (Eq. 20) and causing the catechol content of the reaction mixture to accumulate, shorten the induction period.

The removal of the o-quinone as it is formed at the beginning of the enzymatic oxidation of phenol would, according to Eq. 21, also prevent the formation of the second molecule of catechol, and thereby hinder the autocatalytic rise in the rate of oxidation during the induction period. As previously stated, benzenesulfinic acid removes the o-benzoquinone from the reaction mixture by irreversibly forming dihydroxydiphenylsulfone. When Bordner and Nelson added the sulfinic acid, at the beginning of the reaction, when p-cresol was oxidized by the enzyme, they found that not only was the induction period lengthened but the rate of oxidation of the p-cresol decreased. Pugh and Raper (68) had previously reported that phenol could not be enzymatically oxidized by tyrosinase when benzenesulfinic acid was an initial component of the system. Later, Behm and Nelson (11) showed that the sulfinic acid retarded but did not stop the phenol oxidation when it was added after

the reaction was under way. Furthermore, they found that the addition of a small amount of catechol overcame the inhibiting effect of the benzene-sulfinic acid at the start of the reaction.

The fact that the phenol oxidation appears to be more sensitive than the *p*-cresol oxidation to the influence of the sulfinic acid in depriving the enzyme of the second molecule of catechol raises the following question: Must more catechol be oxidized to activate the enzyme–phenol complex than the enzyme–*p*-cresol complex? The answer seems to be in the affirmative. Behm and Nelson (11) have shown that, when the two monohydric phenols were oxidized by means of the enzyme in the presence of boric acid at *p*H 6, the phenol reaction was retarded more by the boric acid than was the *p*-cresol reaction. It is well known that boric acid tends to combine with *o*-dihydric phenols. Therefore, if the phenol oxidation requires the oxidation of more catechol than the *p*-cresol oxidation does of homocatechol, then the removal of the *o*-dihydric phenol by the boric acid should retard the phenol oxidation the most.

2. The Constant Rate of Oxidation

It was pointed out in Sections II and V that, in the isolation of tyrosinase from its natural sources, and in its subsequent purification, the loss in the enzyme's monohydric acitivity, depending on the procedure followed, is often much greater than the loss in the enzyme's *o*-dihydric phenol activity. It has therefore been found convenient to group different preparations, depending on their ratios of the two activities, into two groups, high cresolase and high catecholase preparations (Section II). These two types of preparations exhibit certain differences when they catalyze the oxidation of monohydric phenols (11, 36, 52). After the enzymatic oxidation of *p*-cresol or phenol has passed the induction period, the rate of oxygen uptake remains constant for a considerable length of time, and then gradually decreases. Curve I in Figure 3 represents the rate of oxygen uptake when a given amount of phenol was oxidized in a Warburg respirometer by means of a high cresolase preparation. Curve II represents the rate when a high catecholase preparation, possessing about the same amount of phenolase activity but more catecholase activity, was used under similar conditions. It will be noticed that the linear portion of Curve II is much longer than that of Curve I. It has been pointed out, in Sections IV and V, that one of the most striking characteristics of the enzymatic oxidation of catechol is the pronounced inactivation of the enzyme during the reaction. A given amount of the enzyme can oxidize only a definite amount of catechol, during which time it becomes irre-

versibly inactivated. Since the phenol oxidation is dependent on the continuous activation of the enzyme–phenol complex by the simultaneous oxidation of catechol by the enzyme, inactivation of the latter must, depending on the amount of catecholase present in the reaction mixture, sooner or later impair the rate of oxidation of the phenol. Therefore, in the case of the long linear portion of Curve II above, it would appear that the high catecholase preparation contained such an excess of catecholase
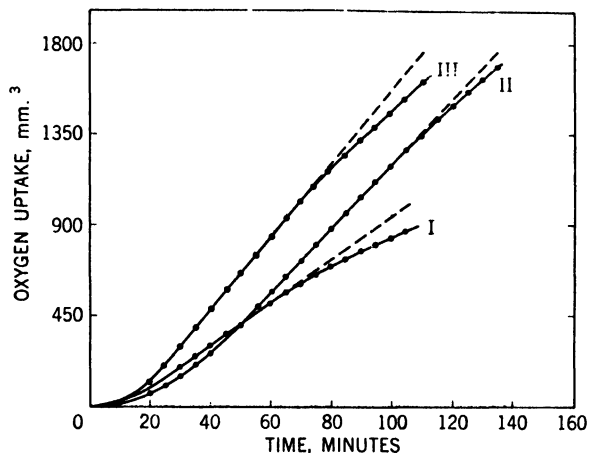
Fig. 3.—The lengths of the linear portions of the curves representing the rates of oxygen uptake when phenol was oxidized by means of a high-cresolase preparation and by means of a high-catecholase preparation of mushroom tyrosinase at 25° C. and pH 6.8.

Reaction mixtures: *Curve I.*—4 ml. water, 2 ml. phosphate buffer, 1 ml. aqueous solution of phenol (22 mg.), and 1 ml. high-cresolase preparation (1.06 phenolase units). *Curve II.*—Same as for Curve I, except that 1 ml. of a high-catecholase preparation containing 1.44 units of phenolase was used. *Curve III.* Same as reaction mixture for Curve I, except that 1 ml. enzyme solution, made up of equal amounts of the high-cresolase and high-catecholase preparations was used (Behm and Nelson, 11).

that the latter was able to maintain an activated enzyme–phenol complex for a considerable period of time even though the catecholase was being gradually inactivated. In the case of the high cresolase preparation (Curve I), the shorter length of the linear portion of the curve would imply that inactivation of the smaller amount of catecholase present in the high cresolase preparation soon resulted in an incomplete activation of the enzyme–phenol complex.

That the linear part of the oxidation curve is a function of the cate-cholase content of the enzyme preparation can also be demonstrated by varying the concentration of the substrate instead of using different preparations of the enzyme. The activity of tyrosinase preparations varies with the concentration of the substrate, and the maximum activity corresponds to a definite optimum concentration (37). Behm and Nelson compared the rates of oxygen uptake when 20, 10, and 5 mg. of phenol were oxidized by a given amount of a high cresolase preparation. Their
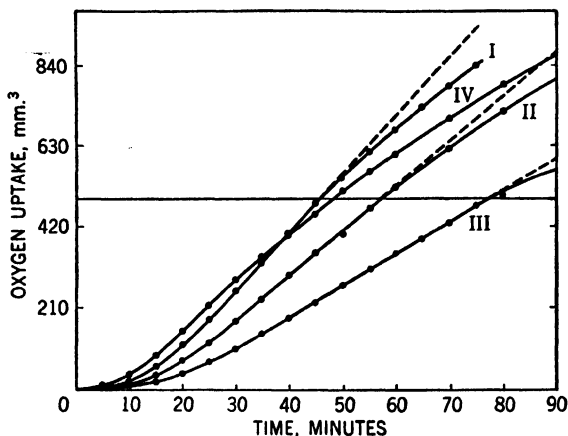
Fig. 4.—The amount of oxygen uptake before the rate starts to depart from a linear course is independent of the concentration of phenol when the latter is oxidized by means of a given amount of a high cresolase preparation of mushroom tyrosinase at 25° C. and pH 6.8.

Reaction mixtures: 3 ml. water, 2 ml. phosphate buffer, 2 ml. phenol solution (phenol content given below), and 1 ml. enzyme solution, containing 1.53 phenolase units. Phenol content of the different reaction mixtures: *Curve I*—20 mg., *II*—10 mg., *III*—5 mg., and *IV*—40 mg. The optimum phenol concentration for the enzyme preparation was 20 mg. in an 8-ml. reaction mixture (Behm and Nelson, 11).

results are shown in Figure 4. It will be observed that all the rate curves start to deviate from a straight line at about the same volume of oxygen uptake. Since for every three atoms of oxygen consumed two atoms are used up in the oxidation of catechol, it follows that at the same oxygen uptake, in each of the three experiments, the same amount of catechol had been oxidized and hence the same amount of catecholase inactivated. Therefore, it would be expected that the stage at which enough active catecholase no longer remained to activate the enzyme–phenol complex

completely would occur in each experiment at the same volume of oxygen uptake.

3. Affinities of Different Phenols for Tyrosinase

It is generally agreed that, for an enzyme to catalyze a reaction, it must first combine with the substrate. In this way, tyrosinase is assumed to combine with phenolic bodies to form phenol–enzyme complexes. The tendency for phenols to combine with the enzyme is dependent, as has been inferred in Section V, on several factors such as the concentration of the phenolic compound, the structure of the phenol, and the temperature and pH of the reaction mixture. The kind of tyrosinase preparation, whether a high cresolase or high catecholase, makes a difference. The degree of purity of the preparation also is an important factor in determining the amount of enzyme–phenol complex formed. High cresolase preparations show higher optimum substrate concentrations than do high catecholase preparations. Roth, Miller, and Dawson determined the optimum concentrations for the maximum rates of oxidation for several derivatives of catechol, using a highly purified high catecholase preparation at pH 5.5 and at 25° C. (see Table II).

Other substances in the reaction mixture often exert a retarding action on the rate of oxidation of the phenolic compound. It has been shown by Behm and Nelson (11) that benzenesulfinic acid behaves as a competitive inhibitor in the enzymatic oxidation of phenol. Ludwig and Nelson (46) report that benzoic acid greatly retards the tyrosinase oxidation of catechol. On the other hand, Miller and Dawson (51) found l-ascorbic acid exerts no retarding action when catechol is oxidized by means of the enzyme. Soloway (79) found d-iminoascorbic acid, d-glucoascorbic acid and reductone, like l-ascorbic acid, do not affect the rate of oxidation of 4,5-dimethylcatechol by tyrosinase.

The rate of oxidation observed when a mixture of two substrates is used depends on the relative competition of the substrates for the enzyme. Behm has found that, when 20 mg. of catechol was oxidized by means of a high catecholase preparation, the rate of oxidation was decreased 60% by the presence of 40 mg. of phenol. The oxidation of 10 mg. of 4,5-dimethylcatechol, on the other hand, was lowered 60% by only 0.05 mg. of xylenol (4,5-dimethylphenol).

When the concentration of the substrate exceeds its optimum there may be a marked decrease in the rate of oxidation, depending on the type of enzyme and type of substrate. For example, Soloway (79), using a high catecholase preparation, found that 0.5 mg. of 4,5-dimethylcatechol in 3 ml. of reaction mixture gave rise to the maximum rate of oxidation. When the concentration was increased to 2 mg. per 3 ml., the rate dropped 60%. On the other hand, Curve IV in Figure 4 shows that the rate of oxidation of 40

mg. of phenol in an 8-ml. reaction volume by a high cresolase preparation was only slightly less than the rate when an optimum concentration of 20 mg. of phenol was used (compare with Curve I).

VII.   Tyrosinase and Plant Respiration

That oxidases may be involved in respiration has been held as possible for a long time.   How to demonstrate experimentally that this is true has, however, been difficult.   One of the obstacles, in the case of tyrosinase, must be attributed to the way our knowledge of the oxidase has developed. As mentioned in the introduction, Onslow held that oxygenase (tyrosinase) was only active toward certain polyhydric phenols, and not toward mono-hydric.   This view, emphasizing the polyphenolase character of the enzyme, has been favored by many recent workers, including Kubowitz, Keilin and Mann, Califano and Kertész, Dixon, and Tenenbaum and Jensen.   Others, including Raper and coworkers, have challenged this view.   Another obstacle was Onslow's demonstration (57) that catechol and catechol derivatives, such as caffeic acid, protocatechuic acid, etc., occurred widely spread in plants.   Her observation focused the attention of workers, interested in the possibility that tyrosinase plays the role of a terminal respiratory oxidase, on the probability that catechol or one of its derivatives plays the role of the hydrogen carrier adjacent to the oxidase in the respiration chain.

Boswell and Whiting (18) were among the first to attempt to show that polyphenol oxidase (tyrosinase) can function as a respiratory enzyme. (Boswell and Whiting prefer the terms "polyphenol oxidase" or "catechol oxidase" rather than "tyrosinase" for the oxidase of the potato.)

They studied, by means of the Warburg respirometer, the rates of oxygen uptake and evolution of carbon dioxide when thin potato slices were permitted to respire in the presence of water buffered with phosphate to pH 5.5.   In this way, they found that the rates of oxygen uptake and carbon dioxide evolution remained practically constant for several hours, and that the respiratory quotient was close to unity.   With the addition of catechol, there was a sudden marked increase in the rate of oxygen uptake.   This increased rate, however, was only of short duration and was followed by a gradual drop, culminating finally in a value of about one-third of the rate shown by the slices respiring in the presence of phosphate only.   Adding more catechol to the reaction mixture after the final low respiration rate had been reached, gave no further new increase in the rate of oxygen consumed, showing that the oxidase was no longer active.   Boswell and Whiting therefore concluded that "a system involving an oxidase, a phenolic compound, and a dehydrase is concerned in two-thirds of the total respiratory gaseous exchanges of the slices of potato tuber, those of the remaining one-third are dependent on some other system."

The main support for the above conclusion rests on the inactivation of the oxidase by the oxidation of the added catechol, since Boswell and

Whiting held that the inactivation of the potato catechol oxidase was a specific action of *o*-benzoquinone. Realizing that *o*-benzoquinone is not a specific inactivator for the enzyme, but believing that the conclusion of Boswell and Whiting was sound, Baker and Nelson (9*a*) repeated the experiments using, instead of catechol, which results in serious inactivation of the enzyme (Section V), 4-*tert*-butylcatechol. The latter exerts practically no inactivating action (75). By its use, they found that the rate of oxygen uptake dropped fully as far below the normal rate of respiration of the slices as when catechol was used, even though the enzyme in the slices still remained active. This drop in the rate of oxygen uptake, therefore, cannot be accounted for by the inactivation of the oxidase, but must be attributed to some other cause. Hence, the claim made by Boswell and Whiting that two-thirds of the respiration of the slices is dependent on the oxidase loses its main support.

Another weak point in their attempt to show that tyrosinase functions as a respiratory enzyme was their use of catechol as the hydrogen carrier adjacent to the terminal tyrosinase in the respiratory chain. The enzymatic oxidation of catechol is too rapid, and the subsequent part of the respiratory chain is unable to reduce the *o*-quinone as fast as it is formed. This is shown by the fact that, when catechol is added to the respiring slices, they soon become highly colored because of the accumulation of quinone or its oxidation product, polymerized hydroxyquinone. To avoid this too rapid oxidation of the hydrogen carrier adjacent to the oxidase, Baker and Nelson added protocatechuic acid, instead of catechol, to the respiring slices. Protocatechuic acid is oxidized by tyrosinase less rapidly than is catechol. Hardly any discoloration of the slices was exhibited, and only a slight drop occurred in the increased rate of oxygen uptake caused by the added acid. This increased rate of oxygen uptake persisted for several hours, in contrast to the rapid and larger increase in oxygen uptake followed immediately by a drop in the rate to only one-third of the normal rate when catechol was used. Furthermore, this increased rate of oxygen uptake due to the added protocatechuic acid was accompanied by a similar rise in the rate of evolution of carbon dioxide, and the R.Q. still remained close to unity. The latter condition could hardly have occurred unless tyrosinase plays the role of a respiratory enzyme.

l-Tyrosine and the Natural Substrate for Tyrosinase

To gain information concerning the substance present in the potato which serves as the natural substrate for the oxidase, Boswell and Whiting

isolated a gumlike material from an alcoholic extract of the potato. When the extract, containing the material, was added to the respiring potato slices, a considerable rise in the rate of oxygen uptake and carbon dioxide evolution took place. This rise remained constant for several hours and therefore indicated that the extract contained the natural substrate for the potato oxidase. The gumlike material obtained from the extract was soluble in water, was precipitated by lead acetate, and gave a color reaction with ferric chloride. They were, however, unable to purify the substance and identify its chemical nature.

Robinson and Nelson (74) also undertook to investigate the nature of the natural substrate for potato tyrosinase. For the purpose, they ground potatoes into a dilute solution of lead acetate heated to 60° C. On filtering the mixture, most of the material which was acted on by tyrosinase was found to occur in the filtrate. Upon purification, the substance turned out to be *l*-tyrosine. The presence of tyrosine in the juice of the potato was observed as early as 1879 by Schultze (78), and recently by Isherwood (42), and also Schmalfuss and Bumbacher (76) have reported the presence of free *l*-tyrosine in potato extract. Although tyrosinase received its name because it catalyzes the oxidation of tyrosine, offhand it may be difficult to see how tyrosine can play the role of a hydrogen carrier in a respiration chain. A hydrogen carrier must be capable of reversible oxidation-reduction. Raper (70) has shown that the first enzymatic oxidation product of *l*-tyrosine is 3,4-dihydroxyphenylalanine, and this reaction is not reversible. When Robinson and Nelson added either potato or mushroom tyrosinase to a reaction mixture containing *l*-tyrosine and ascorbic acid, they noticed that practically no tyrosine was oxidized until all the ascorbic acid had been oxidized to the dehydro state. After the ascorbic acid had been completely oxidized, the oxidation of the tyrosine to a black, melaninlike product then took place. Since the tyrosinase preparations used showed no activity toward ascorbic acid, the oxidation of the latter must be attributed to some other product formed in the reaction mixture. The simplest explanation seems to be that the dihydroxyphenylalanine, the first oxidation product of tyrosine, has a strong affinity for the enzyme and prevents the latter from combining with a fresh lot of tyrosine. As soon as the dihydroxyphenylalanine is enzymatically oxidized to the corresponding *o*-quinone, the enzyme is set free and can take on a fresh supply of tyrosine. In the presence of ascorbic acid, however, the *o*-quinone is immediately reduced back to the dihydroxy form, and thus, as long as any ascorbic acid remains in the reaction mixture, the enzyme becomes continuously occupied by the dihydroxyphenylalanine

and no oxidation of the tyrosine can take place. In other words, when ascorbic acid is present in a reaction mixture containing tyrosine and tyrosinase, only a trace of the tyrosine is oxidized to dihydroxyphenylalanine. The latter, in turn, functions as a shuttle, transmitting oxygen to the ascorbic acid. In the respiration of the plant, other reducing agents, such as the preceding part of the respiration chain, may play a role similar to that attributed to the ascorbic acid.

o-Quinone $+ H_2O \longrightarrow$ melanin products

Preceding part of the respiration chain $\equiv$ 2H $\qquad$ *tyrosinase* $+ O_2$

l-Tyrosine $+ O_2 \xrightarrow[\text{tyrosinase}]{}$ dihydroxyphenylalanine
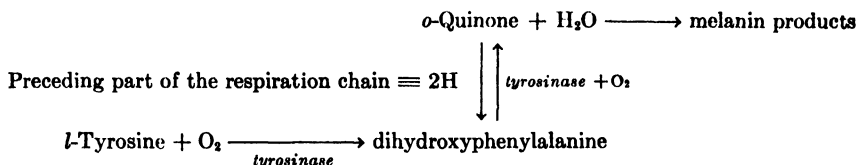
According to the accompanying scheme, 3,4-dihydroxyphenylalanine (dopa) constitutes the true hydrogen carrier, operating adjacent to the terminal oxidase, tyrosinase, in plants such as the potato tuber. l-Tyrosine serves as a reservoir for maintaining the plant cells supplied with the carrier. Any break in the respiration chain, so that the reduction of the quinone back to dihydroxyphenylalanine is interrupted, immediately enables the enzyme to continue to oxidize tyrosine to its final dark, melaninlike oxidation product. Probably the reaction taking place in the discoloration of sliced or peeled potatoes when exposed to air is due to such an interruption of the reaction converting quinone back to its dihydroxy form.

VIII. The Nature of Tyrosinase

In the previous sections of this review, it has been emphasized that the enzyme tyrosinase possesses as its most distinguishing feature the ability to catalyze the aerobic oxidation of both monohydric and o-dihydric phenols. Since the initial steps in the two oxidations appear to be quite different in character, *i. e.*, one involves the insertion of an hydroxyl group while the other involves the removal of two hydrogen atoms, tyrosinase has been regarded as possessing two different catalytic or enzymatic activities. The fact that the ratio of these two activities can be varied during the process of isolation and purification of the enzyme has led naturally to conjecture as to whether or not tyrosinase is one enzyme protein possessing two activities, or is a mixture of two different enzymes, or one enzyme plus some unknown factor. Before considering this question any further, however, it seems advisable to present some of the physical properties of the enzyme.

1. Some Physical Properties of the Enzyme

During the past two years, an attempt has been made in the authors' laboratory to gain additional information about the nature of mushroom tyrosinase by studying the electrophoretic behavior of crude and purified solutions of the enzyme. The experiments have been conducted at 0-5° C. in solutions buffered between pH 5 and 10. Experiments involving the crude preparations have been carried out in the majority of cases using a large-capacity electrophoresis cell (100 ml., Tiselius type) which permitted division of the cell contents at the completion of the experiment into several fractions large enough to allow for enzyme and protein assays. All experiments involving highly purified tyrosinase have been carried out using a small-capacity Tiselius apparatus (10-ml. cell) equipped to analyze the protein boundaries optically.* The latter apparatus has also been used to obtain supplementary data on the crude preparations. It has been necessary to interrupt the work from time to time and consequently the studies are not yet finished. However, certain interesting facts have come to light, permitting some conclusions and speculations regarding the nature of mushroom tyrosinase.

It has been found with crude preparations, i. e., preparations containing several electrophoretically distinguishable protein components, that the ratio of the two activities varies irregularly from one section of the cell to another after the migration has been stopped. On the other hand, highly purified tyrosinase preparations consisting mainly of one protein component, regardless of whether they are high catecholase or high cresolase in character, show little or no variation in activity ratio after electrophoresis. This difference between crude and purified tyrosinase preparations would suggest that the measured ratio of the two activities in crude tyrosinase is influenced by accompanying proteins. This suggestion parallels the fact that the ratio is most easily changed during the early stages of the chemical purification process. Furthermore, another correlation can be found in the fact that the addition of inert protein, such as gelatin, to purified high cresolase and high catecholase preparations of tyrosinase changes the measured ratios of their two activities (50, 52).

During electrophoresis, some of the inactive protein components of crude tyrosinase move faster than the enzyme and some slower. Above pH 5, the nondialyzable dark-colored component moves rapidly ahead of the enzyme and may be thus separated from the active component. The average mobility of the enzyme shows a continuous increase with increase in pH between 5 and 10. The variation with pH of the mobility of the main component of a purified high catecholase preparation (C172–5AB) is shown in Table III.

---

* All electrophoresis runs in the small-capacity Tiselius apparatus and all ultracentrifuge studies were made by Dr. D. H. Moore, College of Physicians and Surgeons, Columbia University, New York, N. Y.

The mobility (μ) is that of the descending boundary, and the homogeneity is expressed as the per cent of the total protein represented by the active component as determined by optical analysis of the boundary

TABLE III

MOBILITY OF MAIN COMPONENT OF PURIFIED HIGH CATECHOLASE PREPARATION

pH	μ, cm.^{2}/sec./volt	Homogeneity, %
4.97	2.4×10^{-5}	100
7.58	4.6×10^{-5}	93
8.90	7.9×10^{-5}	90

during electrophoresis at the pH indicated. The results show that the isoelectric point of the active component is considerably below pH 5, possibly in the vicinity of pH 3 or lower (Kubowitz (45) reported that his purified potato tyrosinase had an isoelectric point at pH 5.4), and that nonhomogeneity in such preparations can be detected electrophoretically more easily at pH 9 than at pH 5. It will be recalled that the enzyme is stable at pH 9, but rapidly loses activity in solutions buffered below pH 5. For this reason, no electrophoresis studies have yet been made in solutions buffered below pH 5.

TABLE IV

ACTIVITY AND ELECTROPHORETIC DATA FOR THREE PURIFIED TYROSINASE PREPARATIONS OF THE HIGH CATECHOLASE TYPE*

Enzyme preparation	Cu %	Activity/γ Cu		Activity/mg. dry wt.		Ratio	Electrophoresis data		
		Cat.	Cres.	Cat.	Cres.	Cat./cres.	μ	pH	Homogeneity, %
C211–228F2	0.21	2130	48	4400	95	48	6.3×10^{-5}	7.58	100
C175–BI	0.10	2100	104	2150	107	20	5.3×10^{-5}	7.71	95
C172–5AB	0.10	2300	137	2270	135	17	4.6×10^{-5}	7.58	93

* Data for preparations from the mushroom, *P. campestris* (Mallette, Ames, and Dawson, 47). Catecholase activity units are those of the chronometric method (Section IV, Eq. 17). The activity values per mg. dry weight when multiplied by 600 give Q_{O_2} values.

The catecholase activity of a purified high catecholase preparation of mushroom tyrosinase is proportional to the copper content of the preparation, and the ratio of catecholase to cresolase activity is high (Table IV).

Some of the electrophoresis results obtained with three different preparations of this type are also shown in the table.

It can be seen from the table that the electrophoretic mobility of the enzyme increases with increase in ratio of the catecholase to cresolase activity, *i. e.*, the surface character of the molecule, upon which the mobility is mainly dependent (1), varies with the ratio of the two activities. Although only a few experiments have been conducted with a purified high cresolase preparation, the results have been in line with this observation. The preparation had an activity ratio of 1.6 and its main component, constituting 70–80% of the total protein as determined electrophoretically, had a mobility of 2.9 $\times$ 10^{-5} cm.^{2}/sec./volt at pH 7.71. A sample of this component was found to have the same activity ratio as the original preparation; the faster moving component, constituting 20–30% of the total protein, had no activity.

The fact that all three of the purified high catecholase preparations (Table IV) were found to be almost electrophoretically homogeneous between pH 5 and 10, even though the copper content and catecholase activity per mg. dry weight varied by a factor of 2, would suggest that the enzymes of the lower copper content contained about 50% by weight of inactive copper-free protein having about the same electrophoretic properties as the active copper–protein enzyme. The active and inactive proteins might or might not have the same mass, for it is well known (1) that the electrophoretic mobility of particles may be quite independent of their mass. As a matter of fact, the most highly purified preparation of those shown in the table (C211–228F2), using the activity and copper content as the basis of purity, was found to be only about 85% homogeneous in the ultracentrifuge (see footnote, page 146), and the light-weight component (about 15% of the total protein) was found to possess no enzymatic activity.

It should be pointed out, however, that the activity, copper, and homogeneity data of Table IV can be correlated equally well in terms of differences in molecular weights of the copper protein enzyme. The active component of C211–228F2 was found to have a molecular weight of about 100,000 (S_{20} = 6.4 $\times$ 10^{-13}; D_{20} = 6.1 $\times$ 10^{-7}). Since the preparation contained 0.21% copper and the active component constituted about 85% of the total protein, it might be inferred that the pure active component of the preparation contained about 0.25% copper. This copper content corresponds to four atoms of copper per enzyme molecule having a molecular weight of 100,000. It follows that a four-copper-atom enzyme molecule having a molecular weight of about 200,000 would contain about

0.13% copper. Unfortunately, it was not possible to determine in the ultracentrifuge the homogeneity and molecular weight of the two high catecholase preparations containing 0.1% copper. However, a molecular weight of 200,000 for mushroom tyrosinase, as determined by the diffusion method of Northrop and Anson (56), was previously reported by Tenenbaum (83). Although the copper content of the preparation used by Tenenbaum was not reported, the fact that the preparation apparently had a considerably lower catecholase to cresolase activity ratio than C211–228F2 is of interest.

2. Conclusions

In view of the data that have been presented above and in previous sections of this report, the authors are of the opinion that, at the present time, the most reasonable interpretation of the physical nature and dual action of the enzyme tyrosinase is as follows. Tyrosinase is one copper protein entity or complex possessing two enzymatic activities. The ratio of these activities depends on factors such as the size, shape, surface characteristics, etc., of the protein complex. A change in ratio during preparative stages would thus be interpreted as being due to a chemical or physical modification or fragmentation of the protein complex. The principal facts or observations supporting this view of the nature of the enzyme are:

1. Depending on the mode of preparation, two types of tyrosinase having quite different catecholase to cresolase activity ratios can be isolated and purified from the same source of mushrooms; but in no case as yet reported has a preparation been obtained having only monophenolase action.

2. Only the catecholase activity of the purified high catecholase preparation is proportional to the copper content; and the purified enzyme has properties that are quite different from the enzyme as it exists in the plant juice.

3. Both activities of purified high cresolase preparations are proportional to the copper content; and such preparations resemble the enzyme as it exists in the plant juice.

4. In all cases, the cresolase activity is dependent on, and closely associated with, the catecholase activity of the enzyme preparation.

5. Purified high catecholase and high cresolase preparations exhibit kinetic, stability, substrate, inactivation, and electrophoresis differences, suggesting that the two types of tyrosinase differ in size or surface character of the protein molecule.

Bibliography

1. Abramson, H. A., Moyer, L. S., and Gorin, M. H., *Electrophoresis of Proteins*. Reinhold, New York, 1942.
2. Adams, M. H., and Nelson, J. M., *J. Am. Chem. Soc.*, **60**, 2472 (1938).
3. Adams, M. H., and Nelson, J. M., *ibid.*, **60**, 2474 (1938).
4. Allen, T. H., and Bodine, J. H., *Proc. Natl. Acad. Sci. U. S.*, **27**, 269 (1941).
5. Allen, T. H., and Bodine, J. H., *Science*, **94**, 443 (1941).
6. Allen, T. H., Otis, A. B., and Bodine, J. H., *Arch. Biochem.*, **1**, 357 (1943).
7. Allen, T. H., Ray, O. M., and Bodine, J. H., *Proc. Soc. Exptl. Biol. Med.*, **39**, 549 (1938).
8. Arnow, L. E., *J. Biol. Chem.*, **120**, 151 (1937).
9. Bach, A., and Chodat, R., *Ber.*, **35**, 2466 (1904).
9a. Baker, D. L., and Nelson, J. M., *J. Gen. Physiol.*, **26**, 269 (1943).
10. Ball, E. G., and Chen, T. T., *J. Biol. Chem.*, **102**, 691 (1933).
11. Behm, R. C., and Nelson, J. M., in press (*J. Am. Chem. Soc.*).
12. Bertrand, G., *Compt. rend.*, **121**, 166 (1895).
13. Bertrand, G., *ibid.*, **122**, 1215 (1896).
14. Bertrand, G., *ibid.*, **145**, 1352 (1908).
15. Bhagvat, K., and Richter, D., *Biochem. J.*, **32**, 1397 (1938).
16. Bodine, J. H., and Tahmisian, T. N., *Arch. Biochem.*, **2**, 403 (1943).
17. Bordner, C. A., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 1507 (1939).
18. Boswell, J. G., and Whiting, G. C., *Ann. Botany*, **11**, 847 (1938).
19. Bougault, M. J., *Compt. rend. soc. biol.*, **49**, 455 (1897).
20. Bourquelot, E., and Bertrand, G., *ibid.*, **47**, 582 (1895); *J. Pharm. Chim.*, **3**, 177 (1896).
21. Bull, H. B., and Neurath, H., *Chem. Revs.*, **23**, 391 (1938).
22. Califano, L., and Kertész, D., *Nature*, **142**, 1036 (1938).
23. Chodat, R., *Abderhalden Handbuch der biol. Arbeitsmethoden.* Abt. 4, Teil 1, Urban und Schwarzenberg, Berlin, 1936.
24. Chodat, R., *ibid.*, Abt. 3, Teil 1, p. 42.
25. Cross, C. F., Bevan, E. J., and Heiberg, T., *Ber.*, **33**, 2015 (1900).
26. Cruess, W. V., and Joslyn, M. A., *Proc. Inst. Food. Tech.*, **1942**, p. 99.
27. Dalton, H. R., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 2946 (1939).
28. Dawson, C. R., and Ludwig, B. J., *ibid.*, **60**, 1617 (1938).
29. Dawson, C. R., and Nelson, J. M., *ibid.*, **60**, 245 (1938).
30. Dawson, C. R., and Nelson, J. M., *ibid.*, **60**, 250 (1938).
31. Dixon, M., *Manometric Methods.* Cambridge Univ. Press, London, 1934.
32. Fieser, L. F., and Peters, M. A., *J. Am. Chem. Soc.*, **53**, 793 (1931).
33. Chapter by Franke, W., in Nord-Weidenhagen, *Handbuch der Enzymologie.* Vol. 2, Akadem. Verlagsgesellschaft, Leipzig, 1940, p. 731.
34. Gessard, C., *Compt. rend. soc. biol.*, **54**, 1304 (1902).
35. Graubard, M., and Nelson, J. M., *J. Biol. Chem.*, **111**, 757 (1935); **112**, 135 (1935).
36. Gregg, D. C., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 2500 (1940).
37. Gregg, D. C., and Nelson, J. M., *ibid.*, **62**, 2506, 2510 (1940).
38. Haehn, H., in Oppenheimer, C., and Pincussen, L., *Die Methodik der Fermente.* Thieme, Leipzig, 1929, p. 1364.

39. Happold, F. C., and Raper, H. S., *Biochem. J.*, **19**, 92 (1925).
40. Harlay, M. V., *J. Pharm. Chim.*, **9**, 468 (1899).
41. Hinsberg, O., and Himmelschein, A., *Ber.*, **29**, 2028 (1896).
42. Isherwood, F. A., *Food Invest. Board Rept. Gr. Brit.*, **1937**, p. 184.
43. Kastle, J. H., "The Oxidases," *Hygienic Lab. Bull.* **59**, U. S. Treasury Dept., Washington, D. C., 1910.
44. Keilin, D., and Mann, T., *Proc. Roy. Soc. London*, **B125**, 187 (1938).
45. Kubowitz, F., *Biochem. Z.*, **292**, 221 (1937); **299**, 32 (1938).
46. Ludwig, B. J., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 2601 (1939).
47. Mallette, M. F., Ames, S., and Dawson, C. R., *unpublished work*.
48. Martinon, M., *Bull. Soc. Chim.*, [2] **43**, 155 (1885).
49. Milas, N. A., *Chem. Revs.*, **10**, 295 (1932).
50. Miller, W. H., and Dawson, C. R., *J. Am. Chem. Soc.*, **63**, 3368 (1941).
51. Miller, W. H., and Dawson, C. R., *ibid.*, **63**, 3375 (1941).
52. Miller, W. H., and Dawson, C. R., *ibid.*, **64**, 2344 (1942).
53. Miller, W. H., Mallette, M. F., Roth, L. J., and Dawson, C. R., *J. Am. Chem. Soc.*, **66**, 514 (1944).
54. Narayanamurti, D., and Ayyar, C. V. R., *J. Indian Inst. Sci.*, **12**, 109 (1929).
55. Nobutani, F., *J. Biochem. Japan*, **23**, 472 (1936).
56. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, **12**, 543 (1928–29).
57. Onslow, M. W., *Biochem. J.*, **13**, 1 (1919); **14**, 535, 541 (1920); **15**, 107, 113 (1921); **17**, 216 (1923); **18**, 549 (1924).
58. Onslow, M. W., *Principles of Plant Biochemistry*. Cambridge Univ. Press, London, 1931.
59. Onslow, H., *Proc. Roy. Soc. London*, **B89**, 36 (1917).
60. Onslow, M. W., and Robinson, M. E., *Biochem. J.*, **20**, 1138 (1926).
61. Onslow, M. W., and Robinson, M. E., *ibid.*, **22**, 1327 (1928).
62. Oppenheimer, C., *Die Fermente und ihre Wirkungen, Supplement.* The Hague, 1939, p. 1608.
63. Parkinson, G. G., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 1693 (1940).
64. Pinhey, K. G., *J. Exptl. Biol.*, **7**, 19 (1930).
65. Platt, B. S., and Wormall, A., *Biochem. J.*, **21**, 26 (1927).
66. Pugh, C. E. M., *ibid.*, **23**, 456 (1929).
67. Pugh, C. E. M., *ibid.*, **24**, 1442 (1930).
68. Pugh, C. E. M., and Raper, H. S., *ibid.*, **21**, 1370 (1927).
69. Raper, H. S., *ibid.*, **26**, 2000 (1932).
70. Raper, H. S., *Physiol. Revs.*, **8**, 244 (1928); *Ergeb. Enzymforschung*, **1**, 270 (1932).
71. Reinders, W., and Dingemans, P., *Rec. trav. chim.*, **53**, 209 (1934).
72. Richter, D., *Biochem. J.*, **28**, 901 (1934).
73. Robinson, M. E., and McCance, R. A., *ibid.*, **19**, 251 (1925).
74. Robinson, E. S., and Nelson, J. M., *Arch. Biochem.*, **4**, 111 (1944).
75. Roth, L. J., Miller, W. H., and Dawson, C. R., *unpublished work*.
76. Schmalfuss, H., and Bumbacher, H., *Biochem. Z.*, **315**, 97 (1943).
77. Schoenbein, C. F., *Phil. Mag.*, **11**, 137 (1856).
78. Schultze, E., *Ber.*, **12**, 1924 (1879).
79. Soloway, S., *Dissertation*, Columbia University, 1941.
80. Sutter, H., *Ergeb. Enzymforschung*, **5**, 273 (1936).

81. Szent-Györgyi, A., *Biochem. Z.*, **162**, 399 (1925).
82. Szent-Györgyi, A., and Vietorisz, K., *ibid.*, **233**, 236 (1931).
83. Tenenbaum, L. E., *Dissertation*, Columbia University, 1940.
84. Tenenbaum, L. E., and Jensen, II., *J. Biol. Chem.*, **147**, 27 (1943).
85. Von Fürth, O., and Schneider, H., *Beitr. Chem. Physiol. Path.*, **1**, 229 (1901). (Taken from reference 43.)
86. Wagreich, II., and Nelson, J. M., *J. Am. Chem. Soc.*, **60**, 1545 (1938).
87. Wagreich, H., and Nelson, J. M., *J. Biol. Chem.*, **115**, 459 (1936).
88. Yakushiji, E., *Acta Phytochim. Japan*, **10**, 63 (1937).
89. Yoshida, H., *J. Chem. Soc.*, **43**, 472 (1883).

GRAMICIDIN, TYROCIDINE, AND TYROTHRICIN

By

ROLLIN D. HOTCHKISS*

New York, N. Y.

CONTENTS

I. Introduction and Summary

Observations of the antagonistic effect of certain aerobic spore-forming bacteria on the growth of other microorganisms were recorded as long

---

* Lieutenant-Commander, Hospital Corps, U.S.N.R.   The Bureau of Medicine and Surgery of the United States Navy does not necessarily endorse the views or opinions expressed in this article.

ago as 1888. During subsequent years a number of reports described various bacteriolytic and growth inhibitory or "antibiotic" effects brought about by living cells, and often by culture filtrates, of members of this class of bacteria. The "antibiotic agents" responsible for the action of culture filtrates were not purified until, in 1939, Hettche and Weber were able to attribute the antagonistic properties of *Bacillus mesentericus* filtrates for diphtheria organisms to isovaleric and oleic acids produced during growth. In the same year, Dubos described the preparation from cultures of an aerobic spore-forming organism, later identified as *B. brevis*, of a well-defined protein-free concentrate, having the antibacterial properties of the original cultures. From this highly active product, later named *tyrothricin*, were eventually separated by crystallization two alcohol-soluble polypeptides, *gramicidin* and *tyrocidine*. Gramicidin and the parent mixture, tyrothricin, in minute amounts prevent growth of a variety of Gram-positive microorganisms *in vitro* and, under some conditions, also *in vivo*. Tyrocidine, in the absence of serum, extraneous proteins, or peptones is effective in killing both Gram-positive and Gram-negative bacteria, but has little activity when such products are present, as, for example, when used *in vivo*. Tyrothricin has been tested in a considerable number of attempts to modify the course of infections in animals and man, and has in some instances been effective, although its toxicity for the host precludes its general usefulness. The pure substances, gramicidin and tyrocidine, have chiefly been studied chemically and biochemically with the aim of relating their chemical and physical natures to the mechanism of their antibacterial actions. Both polypeptides appear to have molecular weights of 3000 or less and to contain a proportion of *d*- or "unnatural" amino acids. Tyrocidine, as a large molecule of which the principal active groups are basic, has some properties of the cationic detergents; its action on susceptible cells is attended by lytic and autolytic effects, and by the cessation of fermentative and respiratory metabolic processes. Gramicidin has no free basic or acidic groups; perhaps as a consequence it is less drastic than tyrocidine in its action. It is found to be primarily growth inhibitory, or bacteriostatic, rather than directly lethal, for bacteria. Cells affected by gramicidin remain intact and retain the ability to perform many of their normal metabolic processes. However, there is interference with some process or some few processes by means of which, in the normal cell, the energy derived from metabolic activity performs the useful work of chemical transport and synthesis.

The purpose of this review is to describe, insofar as possible the investigations which have led to the conclusions just summarized.

II. Microbial Antagonism by Aerobic Spore-Forming Bacteria

The historical side of microbial antagonism has been thoroughly reviewed by Waksman, who tabulates references to some 25 articles recording effects by the class of aerobic spore-forming bacteria alone, and many more concerned with other classes of bacteria (162).

It seems probable that one of the earliest reports, to the effect that *Bacillus anthracis* and typhoid organisms grew only feebly in filtrates of *B. anthracis* cultures four to six weeks old (44), can be explained as simple exhaustion of the medium in one or more growth substances, which of course might have a specific effect on certain species. The antagonism by anthrax organisms found by Sirotinin in another early work (146) was irregular, and in the author's own conclusion, negligible. The observations of Nicolle in 1907 (113) and of Rosenthal in 1925 (136) that *B. mesentericus* and *B. subtilis* culture filtrates, and of Much and others from his laboratory during 1923–1926 (43, 81, 107, 108, 140) that *B. mycoides* culture filtrates caused the lysis of many species of microorganisms, are however, clear-cut evidence of toxic factors elaborated by the growing bacteria. It is possible that the effects are explained by the demonstration, by Hettche and Weber, that isovaleric and oleic acids are produced by *B. mesentericus* in sufficient quantities (concentration 1:500, 1:7500 or more, respectively) to be bactericidal for diphtheria bacteria (60). These last workers reported that the ether-insoluble portion of the filtrates was virtually inactive, and also that higher concentrations of the pure acids were toxic for other than diphtheria organisms. However this may be, it appears likely that fatty acids account for the growth inhibition of diphtheria bacteria by *B. mesentericus* cultures (3, 9, 123) and culture filtrates (165, 171), conceivably also for inhibition by *B. subtilis* (9).

Since Hettche and Weber were the only ones to report any chemical fractionation of culture filtrates, it is impossible to know whether toxic principles other than fatty acids were present in the cultures studied by the earlier workers. Perhaps the broader toxicity found by Pringsheim, including growth inhibition of *Proteus vulgaris*, *B. mycoides*, and *Neisseria meningitidis*, as well as of diphtheria bacteria, is indicative of more complex toxins elaborated by the spore-forming organisms. The observations of van Canneyt that *B. subtilis* can overgrow and kill tubercle bacilli (16), and of Sanfelice that "*B. anthracis colisimile*" cells kill *B. anthracis* (139), are other examples of antagonism which may be due to such diverse phenomena as acid or toxin production, competition for growth factors, or direct enzymatic attack on one organism by another.

Miscellaneous observations include effects of *B. subtilis* filtrates on trypanosomes (86) and rabies virus (98), and of the living cells on the growth of *Hemophilus influenzae* (168). In addition, several workers have found related organisms to be toxic for various fungi (for references, see 162 and 17).

In 1939, Dubos reported isolation from soil of an aerobic spore-forming organism capable of lysing the living cells of many Gram-positive bacteria (29). The original isolation came about as the result of an attempt to find a microbial enzyme able to attack the Gram-positive substance or structure of the pyogenic cocci; but Dubos soon demonstrated that a more or less stable, nitrogenous, alcohol-soluble toxin which the organisms released on autolysis accounted for the bactericidal effect (32). Although it is the later study of this agent that will occupy the greater part of our attention, some of the subsequent biological work should be mentioned at this point.

Hoogerheide had for some time been occupied with the study of capsule production by the Gram-negative Friedländer's bacillus, and had isolated from soil a microorganism which inhibited the formation of capsular material without preventing growth (67). Being struck with the resemblance of his inhibiting organism to that found by Dubos, he soon showed that its culture also contained a bactericidal substance and that other strains could be easily obtained from soil with similar action (68). McDonald states that the staff of the Biochemical Research Foundation obtained smaller yields of similar bactericidal products from untrained or stock strains of the sporulating bacteria, *B. subtilis*, *B. megatherium*, *B. mesentericus*, and *B. cereus* (101). Dubos and Hotchkiss reported the identification of Dubos' spore-forming organism as *B. brevis* (strain BG), and the isolation from cheese, soil, manure, and sewage of several other strains, all of which produced varying amounts of similar alcohol-soluble bactericidal toxins (34). They concluded that Rosenthal's toxic filtrates (mentioned above) had contained the same toxic agents; the *Tyrothrix* strains used by him were identified as *B. subtilis*. Rosenthal was then stimulated to reinvestigate his lytic cultures, and concluded that they lysed only heat-killed Gram-negative organisms and inhibited growth only of certain Gram-positive forms (137). Stokes and Woodward described a convenient technique which enabled them to recover by simple means 24 strains of bactericidal organisms from soil, all of which proved to be spore-forming bacteria (149). The seven strains which were active against Gram-positive bacteria were nonsaccharolytic, liberated hydrogen sulfide in peptone media, and produced alcohol-soluble toxins like those of the original culture of *B. brevis*.

Katznelson (79), using an unidentified strain of this class of bacterium which had been found by Cordon and Haenseler to be toxic for fungi (22), showed that its culture filtrates suppressed growth of many, though not all, Gram-positive organisms. *B. brevis* and *B. mycoides*, but not *B. subtilis*, were among those inhibited. He had earlier shown that the fungistatic substances were diffusible and could be adsorbed on charcoal and eluted by 95% alcohol (78). Similar fungistatic factors were now found to be produced by the related spore formers, *B. subtilis, B. cereus,* and *B. pumilus*, but not by a stock strain of *B. brevis*. Despite some resemblance to the alcohol-soluble agent of Dubos and Cattaneo, these agents may be different from it, since Katznelson's strain of *B. brevis* appeared not to produce appreciable quantities of toxin and, on the contrary, to be inhibited by it. Stokes and Woodward had shown that the toxin-producing sporulating soil bacteria were resistant to their own toxins (149). However, it is also true that the alcohol-soluble toxin produced by the BG strain of *B. brevis* is markedly fungistatic (148).

Ark and Hunt have reported that culture filtrates of *B. vulgatus* and another, unidentified, spore-bearing bacillus are antagonistic toward a wide variety of bacteria and fungi (2). The toxic agents are said to be soluble and relatively thermostable, and do not inhibit growth in some hexose-free peptone media.

It is clear from the above discussion that aerobic, sporulating bacteria have often been found to be antagonistic toward other microorganisms. Probably very many such observations were never recorded in the literature. However, one can say that, except for certain fatty acids, the only well-defined antibacterial agents bacteria of this class have been shown to produce are alcohol-soluble toxins similar to, perhaps identical with, that elaborated in largest amount by certain strains of *B. brevis*.

III. Purification and Chemical Study
of the Antibacterial Substances from *Bacillus brevis*

1. *Tyrothricin, Gramicidin and Tyrocidine*

The bactericidal activity of *B. brevis* was first detected in growing cultures, but filtrates of autolyzed cultures were found to have the action as well. A protein material precipitated at weakly acid reaction from this solution was the first concentrated preparation (29). An alcohol-soluble, water-insoluble fraction from the protein precipitate proved to be much more highly active (32). From this fraction there were obtained two

crystalline polypeptides accounting for much of its activity (74, 77). The names of these pure substances, *gramicidin* and *tyrocidine*, as well as that of the crude alcohol-soluble mixture, *tyrothricin*, were loosely chosen, to recall the now unused name *Tyrothrix* (see above), and the killing power these preparations have for Gram-positive bacteria.
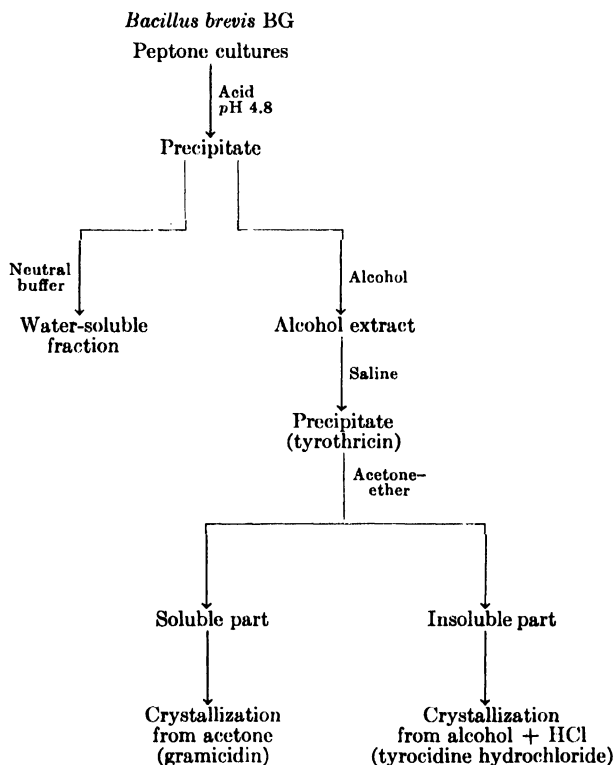
Fig. 1.—Scheme for fractionating the antibacterial substances from cultures of *Bacillus brevis*.

Figure 1 presents a simplified scheme of the fractionation used in preparing these materials. The first steps depend upon the alcohol solubility and water insolubility of the agents. The later steps make use of the lowered solubility of the salt, tyrocidine hydrochloride, in organic solvents containing ether, in order to separate it from the more soluble gramicidin. Although some modifications of this procedure have occasionally been used, all depend upon these simple principles except that of Gordon, Martin, and Synge, who obtained an imperfect separation by chromatography (48).

Reproduced in Figures 2 and 3 (see page 161) are photomicrographs of crystalline gramicidin and tyrocidine hydrochloride. Some physical properties of the two substances are given in Table I. Their solubilities are somewhat unexpected considering their polypeptide natures. When diluted from alcohol solutions into water, dilute glycerol, or glucose, gramicidin gives an opalescent suspension and tyrocidine a clear solution, but both substances are insoluble and flocculated when electrolytes are present.

TABLE I

PHYSICAL PROPERTIES OF GRAMICIDIN AND OF TYROCIDINE HYDROCHLORIDE

Property	Gramicidin	Tyrocidine hydrochloride	References
Melting point.	228–230° C.	240° C. (decomposes)	74, 77, 101, 156
$[\alpha]_D$ in alcohol.	+5°	−101°	49, 77, 156
Soluble in.	Lower alcohols, moist acetone or dioxane, acetic acid, pyridine	Moist alcohols, acetic acid, pyridine	. . . .
Sparingly soluble in.	Dry acetone or dioxane	Dry alcohols, acetone, water	. . . .
Insoluble in.	W a t e r, electrolytes, e t h e r, chloroform, hydrocarbons	Electrolytes, e t h e r, chloroform, hydrocarbons	. . . .
Crystal form.	Platelets with rounded lenticular outline	Needles	74, 77, 101
Crystallized from.	Acetone, dioxane	M e t h a n o l – HCl, ethanol–HCl	74, 77
Absorption maxima, Å. .	2815, 2905, 2710	. . . .	77, 101, 156
Extinction coefficient: $E_{1\ cm.}^{1\%}$ at 2815 Å.	125	. . . .	156

The yield of tyrothricin varies with the different strains of *B. brevis* and with the culture methods. More than 0.5 gram per liter has been produced by the BG strain. The composition and activity of different preparations is remarkably constant, although it has been difficult to isolate a high yield of crystalline gramicidin from some recent commercial preparations. There is reason to believe that tyrothricin normally contains from 10 to 20% gramicidin and 40 to 60% tyrocidine hydrochloride, of which perhaps two-thirds can ordinarily be obtained in crystalline form.

The unaccounted-for portion of tyrothricin appears to be largely made up of "gramicidin-like" and "tyrocidine-like" components, speaking in both a chemical and a biological sense. A fraction crystallizing in clusters of microscopic needles was originally designated as "gramidinic acid" (74); but later, on the basis of its acid–base binding

properties and apparent conversion to tyrocidine hydrochloride, it was assumed to be a partially neutralized form of this latter compound (76, 77). The other early name, "graminic acid," was also withdrawn and replaced by *tyrocidine hydrochloride* when the former designation for this compound was considered inappropriate and likely to be confused with names like *gramine* and *graminin* (an indole base and polysaccharide, respectively, found in members of the grasses, *Gramineae*). Gordon, Martin, and Synge have suggested that there occurs along with gramicidin closely related components of only slightly different composition and solubility (48). The reviewer has often noted that an easily prepared acetone–ether extract of tyrothricin has many of the properties of pure gramicidin although only a part of it can be crystallized. A small quantity of inactive material, including insoluble matter, waxy substances, and a higher fatty acid which is apparently stearic acid are usually encountered as minor constituents of tyrothricin.

What relation the polypeptides have to each other and to the proteins of *B. brevis* or its culture autolyzates is not known. Tyrocidine aids gramicidin to form a more stable dispersion in distilled water, but there is no sign of stable chemical interaction between them. Apparently the active protein found in cultures carries these two water-insoluble substances in a form soluble in water at neutral reaction, and only one-hundredth as active by weight. It is to be noted that the mixture of active polypeptides is precipitated together with the protein at pH 4.5, but separated in water-insoluble form when the protein is heated or treated with proteolytic enzymes (29), or is extracted with alcohol or acetone at acid reaction (32). These properties suggest a labile type of combination; whether or not the protein material carries the polypeptides in high-molecular form or merely solubilizes them cannot be said. The system is somewhat reminiscent of the wheat lipoprotein described by Balls, Hale, and Harris, which, when treated with alcoholic hydrochloric acid, is irreversibly split, undergoing in this case a change from an ether-soluble to a water-soluble form. From the mixture of proteins recovered, one substance was crystallized as a basic protein hydrochloride which had bacteriostatic and fungicidal activity (5).

The formation of tyrothricin is not dependent upon any exacting amino acid composition of the medium in which the *B. brevis* is grown. Media based on tryptone or tryptophane-free gelatin hydrolyzate (34) and synthetic asparagine media (150) in shallow layers have been used successfully. When aerated submerged cultures are employed, tyrothricin does not accumulate in complex nitrogenous media or mixtures of amino acids, even though growth is good. In appropriate aerated synthetic media, however, the total tyrothricin content increases in proportion to bacterial growth and is not related to the extent of autolysis (150). This makes it appear probable that the active polypeptides are constituents of the living bacillus and that, though liberated, they are not produced, by autolysis.

2. The Chemical Nature of Gramicidin

Gramicidin is a neutral substance containing carbon, hydrogen, nitrogen, and oxygen only. It gives positive biuret and indole color reactions but negative color reactions with diazonium salts or Millon's reagent. Acid hydrolysis reveals that it is a polypeptide.

Table II records the published values for the apparent molecular weight or equivalent weight of gramicidin. The solubility of this substance in various organic solvents would appear to favor the physical determination

Fig. 2. - Crystalline gramicidin
(× 190).

Fig. 3.—Crystalline tyrocidine hydrochloride (× 270).

of molecular weight, but the results of these have been at considerable variance. There is no evidence that the original estimate of 1400 from the depression of the freezing point of camphor (75) is invalid, but it is quite possible that decomposition and a low apparent molecular weight may result from heating a complex polypeptide at 175° C. The shift of the specific depression of the freezing point with concentration in cyclohexane solution (156) is direct evidence of some anomalous behavior which certainly invalidates the ordinary calculation of a molecular weight in this solvent. This anomaly has been discussed from the standpoint of hypothetical chemically bound water dissociating reversibly in certain specific

solvents (48).   Whether phenol solutions behave the same way has apparently not been tested; camphor solutions apparently do not.   The isothermal distillation method probably offers the value that is at the moment the least objectionable for the molecular weight of gramicidin.

The available chemical determinations of equivalent weight are not very accurate.   Where an acid hydrolyzate is analyzed, tryptophane recovery would be expected to be low, although there is apparently little destruction when pure tryptophane or gramicidin is heated in strong acid (70).   Hydrolysis with alcoholic alkali (19) gave still less tryptophane and

TABLE II

MOLECULAR AND EQUIVALENT WEIGHTS REPORTED FOR GRAMICIDIN

Method	Ref.	Molecular weight
Cyroscopic:		
camphor	70, 75	1540, 1390, 1385
	101	1340, 1410, 1622
	156	Comparable with above
phenol	156	864, 908
cyclohexanol	156	589–1220, proportional to concentration
Distillation equilibrium:		
butanol	156	In range 2500–4500
methanol	156	3100
Tryptophane content:		
acid hydrolyzate	70	$(n \times) 510$
alkali hydrolyzate	19	$(n \times) 575$
Acetyl equivalent	70	$(n \times) 1200, 1300, 1140, 1240$
Sulfur content of flavianate	156	$(n \times) 2986 \pm 300$
Carbon content of flavianate	156	$(n \times) 3036 \pm 300$

accordingly a higher equivalent weight; but the reviewer has not found this procedure as trustworthy as acid hydrolysis for gramicidin, and is inclined to give more weight to tryptophane analyses performed on the intact substance (71). This procedure, amounting to hydrolysis in the presence of the color reagent at room temperature, has indicated equivalent weights of around 465 in unpublished determinations not included in Table II. Until more is known about the molecule itself and its behavior in acetic anhydride, one cannot be absolutely certain that acetylation furnishes a quantitative estimation of the hydroxyl group. However, it has been pointed out that any acetolysis of amide linkages should have liberated one equivalent of new acetylamino acid for each acetyl residue bound, and

therefore should not affect the result. Again, the nature of the combination with flavianic acid is somewhat obscure and the complex is noticeably dissociated in solution. Calculations based on the analysis of the flavianate involve the assumption of a combining ratio and of the complete attainment of this ratio in an undissociated solid compound. The constancy of analytical results suggests that the latter requirement, at least, is satisfactorily met.

<div align="center">

TABLE III

CONSTITUENTS OF GRAMICIDIN

</div>

Constituent	Nitrogen as % of total nitrogen					Equivalents per 30 N atoms			"Theory" % of total nitrogen
	Ref. 70	Ref. 19	Ref. 48	Ref. 104	Ref. 71	Found	Ref. No.	As- sumed	
After hydrolysis:									
Acidic groups (titration)	..	..	..	..	..	25.4	70	24	..
Basic groups (titration)	80.5	..	..	..	..	24.2	70	24	80.0
Amino N	80.4	71.7	..	..	78.2	24.1	70	24	80.0
α-Amino N	72.6	..	..	..	70.3	21.8	70	22	73.4
Ammonia	1.4	0.3	..	..	0.5	0.2	71	0	0
Aminohydroxy + ammonia	6.0	7.3	..	..	6.8	2.0	71	2	6.7
l-Tryptophane	37.3	32.3	40–45	31+	32.3	4.9	70	6	40.0
d-Leucine	16+	11+	20.0	21.3	14+	6.4	104	6	20.0
dl-Valine	..	..	16.6	..	12+	5.0	48	4	13.3
l-Alanine	30−	23.5	10.1	..	6+	3.0	48	4	13.3
Glycine	..	0	5.9	..	..	1.8	48	2	6.7
Before hydrolysis:									
Tryptophane	..	..	..	..	41.0	6.1	71	6	40.0
Hydroxyl group	..	..	..	..	..	1.1	70	1	..
Acidic and basic groups	0	0	..	..	..	0		0	..

The data apparently indicate that gramicidin is in one of two classes of molecular weight, either around 1300–1500 or 2800–3200. Since these differ by a factor of 2 it will be satisfactory to discuss the composition of a hypothetical molecule having a molecular weight of about 2800. A unit of 30 nitrogen atoms is chosen, since it, like the 15-nitrogen molecule previously treated (70), appears to be most compatible with the more dependable of the analyses for constituents. There is nothing in the purely chemical data to indicate a choice between the larger and the smaller unit.

Analytical results, presented in Table III, do not require much comment. All the investigations from which they come have been somewhat preliminary ones. The recent determinations by Gordon, Martin, and Synge (48) deserve special mention. Their data have been obtained in several runs using in all some 155 milligrams of gramicidin, far less than the 1- and 2-gram samples used by the other workers. The pitfalls of their new method, an ingenious chromatographic adsorption technique, are probably not yet fully known; one limitation is that certain empirical recovery factors have to be used. Nevertheless, it should be obvious that the method is of great promise for investigating such products as gramicidin. The amino acids which are present in this substance, with the exception of tryptophane, are aliphatic ones whose color-producing potentialities and solubilities make quantitative estimation most difficult. The quantities of leucine, alanine, valine, and tryptophane listed with a + sign in the table were actually isolated with the use of some of the aromatic sulfonic acids introduced by Bergmann and coworkers recently as amino acid reagents (8, 27, 147). d-Leucine was determined by the solubility method developed for l-leucine by Moore and Stein (105). This involved measuring the solubility of d-leucine-2-bromotoluenesulfonate in the hydrolyzate after precipitation of the tryptophane as its picryl sulfonate (104). The determination is to be considered as preliminary only, as there has not been opportunity to confirm or extend the results since they were obtained.

With the observation that d-amino acid oxidase acted upon gramicidin hydrolyzates (75, 90), it was concluded that part of the amino acids of the polypeptide were the "unnatural" isomers. This was later confirmed through the isolation of d-leucine (19, 70) and then of dl-valine (35, 71). The tryptophane (19, 70, 90) and alanine (35, 71) were recovered as natural l-isomers. Apparently the only amino acid which might have been racemized during hydrolysis is valine. Since the other amino acids were recovered essentially optically pure, this possibility has considerable interest. Christensen was recently successful in recovering valylvaline containing equal parts of d- and l-valine after brief acid hydrolysis (18). It does not appear possible that the copper salt method of isolation used could have brought about appreciable fractionation. Even if some unique mode of combination of the residues predisposes to racemization during hydrolysis, it is certainly surprising that *both* valine residues in the dipeptide should be so racemized. Gordon, Martin, and Synge have confirmed the reported configuration of all of the constituents by isolating the respective acetylamino acids, but despite recovery of acetyl-dl-valine have envisaged the presence of five total residues per molecule. This would mean either inhomogeneity

of gramicidin or at least partial racemization during hydrolysis since a content of two *l*- and three *d*- residues or vice versa would correspond to only 80% of the racemic form.   The rotation of isolated valine—$[\alpha]_D^{24}$ = +0.3 ± 0.9° in hydrochloric acid (71)—and acetylvaline—$[\alpha]_D$ = 0° ± 0.3° (48)—indicates a minimum of 96% and 92.5% *dl*-valine, respectively. The reviewer has chosen the somewhat lower even number of four residues of valine for Table III, since unpublished results have indicated that at most no more than 15.5% of the total nitrogen can be isolated as valine azobenzenesulfonate.

The assumed number of equivalents per 30 nitrogen atoms in the table is in many cases weighted somewhat arbitrarily, and there is ground for different choices, *e. g.*, 6, 6, 3, 5, 2 and 2 residues of tryptophane, leucine, valine, alanine, and glycine, respectively (48).   More analyses of higher accuracy are needed.   Independently of the exact distribution, however, it is apparent that α-amino acid nitrogen + tryptophane indole nitrogen + aminohydroxy nitrogen + ammonia account for essentially the total content of this element.   About 45% of the α-amino acids present in the hydrolyzate were oxidized by *d*-amino acid oxidase (90) with liberation of 33% of the total nitrogen as ammonia.   The *d*-leucine and *d*-valine assumed to be present account for about 27% of the total nitrogen, perhaps not too low to be within the error of the enzymatic determination.   Since the aminohydroxy constituent has not been characterized, it is not known whether this can be oxidized by the *d*-amino acid oxidase preparation used.

A suitable empirical formula for the assumed 30-nitrogen unit is $C_{148}H_{210}$-$N_{30}O_{26}$ (molecular weight, 2826) or neighboring formulas differing by one or two atoms of carbon or hydrogen.   This is twice the unit discussed previously (70).   Such a molecule would leave from 6 to 10 carbon, 4 oxygen, and 2 nitrogen atoms in the residues remaining unidentified for almost any reasonable disposition of the identified aliphatic amino acid residues. Of this remainder, two amino and two hydroxyl groups are recognized; furthermore, these must be arranged in 1,2-aminohydroxy (or perhaps glycol + diamino) combination on the end of one or more carbon chains (70).   They are not β-hydroxy-α-amino acids (70).   It has not been possible to isolate even small amounts of an aldehydo acid hydrazone after periodate oxidation (71).   This may be because lactone formation has occurred. However, this observation throws doubt on the assumption that the aminohydroxy component is a carboxylic acid, such as isoserine (48, 70).

That no free carboxyl or amino groups remain in gramicidin indicates that amide or ester formation has covered all of them.   Volatile acids and

alcohols have not as yet been found in the hydrolyzate* and fatty acids appear to be absent from pure gramicidin. If such substances are actually present, the molecule may be an extended chain; but at the moment one has to suppose that the ends of the chain are combined to produce a large cyclopeptide, one having 24 —CO—NH— linkages, 22 of which belong to α-amino acids. Of the arrangement in this chain we know only that two valine residues are adjacent (18). Ogston has discussed some of the implications of the possible periodic arrangement of amino acid residues, using gramicidin as an example (115).

The picture of gramicidin as a large cyclopeptide with a high content of tryptophane and aliphatic d- and l-amino acids, if somewhat unexpected, may yet be indicative of other structures which will be discovered among proteins and the little-understood group of natural polypeptides. The eventual determination of its total composition and the arrangement and spacing of its constituents should prove to be of interest to the students of protein structure as well as to those concerned with bacterial toxins.

3.   The Chemical Nature of Tyrocidine

Tyrocidine is a polypeptide with a greater content of reactive groups and complex amino acids. Equivalent weights of 855 by alkali titration, 1285 by chloride analysis, and 815 by easily hydrolyzable ammonia suggested a molecule of 26 nitrogen atoms (70). Ogston has attempted to determine the molecular weight of tyrocidine in dilute acetic acid with the ultracentrifuge. Sedimentation was slow and comparable in magnitude to diffusion; the molecular weight could only be estimated as probably between 1000 and 3000 (116).

The available analyses presented in Table IV are in good agreement with the 26-nitrogen unit. The indicated structure, a polypeptide composed of 3 phenylalanine and 3 ammonia residues and 2 residues each of glutamic acid, aspartic acid, ornithine, tryptophane, tyrosine, proline, leucine, and valine, less 22 molecules of water would have the empirical formula, $C_{127}H_{166}N_{26}O_{26} \cdot 2HCl$, and a molecular weight of 2546. This calculated formula is in good agreement with the elementary analyses, differing by a single carbon atom only from the double molecule previously deduced from

---

* In one hydrolyzate, small quantities of acetaldehyde have been found and identified as the 2,4-dinitrophenylhydrazone (C 42.88%; H 3.35%; iodoform reaction in cold, etc.) (71). The opportunity to confirm the presence or ascertain the origin of the acetaldehyde in another hydrolyzate has not presented itself. If not an artifact of the experimental procedures, it may be involved in blocking terminal groups of a chain.

the elementary composition alone (35, 70). Another formula, containing the elements of one more molecule of water, is also satisfactory:

$$\text{Found:} \quad \text{C, 59.6} \quad \text{H, 6.7} \quad \text{N, 14.3} \quad \text{Cl, 2.76}$$

Calculated: $C_{127}H_{166}N_{26}O_{26} \cdot 2HCl$ 59.9   6.6   14.3   2.78

$C_{127}H_{168}N_{26}O_{27} \cdot 2HCl$ 59.5   6.6   14.2   2.77

TABLE IV

CONSTITUENTS OF TYROCIDINE HYDROCHLORIDE

Constituent	Nitrogen as % of total nitrogen			Equivalents per 26 N atoms			"Theory" % of total nitrogen
	Ref. 70	Ref. 19	Ref. 49	Found	Ref. No.	As-sumed	
Intact tyrocidine:							
Chloride	..	..	..	2.06	70	2	..
Weak acid group	..	..	..	0.98	70	1	..
Amino nitrogen:							
formol titration	8.1	..	..	2.1	70	2	7.7
(Van Slyke) 30 min.	6.8	..	..	1.8	70	2	7.7
(Van Slyke) 140 min.	10.3	..	..	2.6	70	2	7.7
Ammonia, brief hydrolysis	12.0	..	11.8*	3.1	70	3	11.5
Tryptophane	15.9	..	15.5**	2.07	70	2	15.4
Hydrolyzed tyrocidine:							
Acidic groups, titration	..	..	..	24.5	70	23	..
Basic groups, titration	88.6	..	..	23.0	70	24	92.3
Amino groups (corrected)†	..	82	..	21.3	19	21	80.8
α-Amino groups	73.4	..	..	19.1	70	19	73.1
Ammonia	13.3	12.8	..	3.3	19	3	11.5
Dicarboxylic amino acids	16.5	15	13.1	4.2	70	4	15.4
Aspartic acid	*ca.* 4.5	..	6.5	1.7	49	2	7.7
Glutamic acid	..	..	6.6	1.7	49	2	7.7
Basic amino acids	..	15	..	1.95	19	2	15.4
Ornithine	..	..	9–13	1–2	49	2	15.4
Tryptophane	11.7	5–7	1–10	1.5	70	2	15.4
Proline	..	..	6.7	1.7	49	2	7.7
Tyrosine	..	7.1	6.4	1.85	19	2	7.7
Leucine	..	..	8.2	2.1	49	2	7.7
Valine	..	..	7.2	1.9	49	2	7.7
Alanine	..	7.2	0	0	49	0	0
Phenylalanine	..	13	13.2	3.4	19	3	11.5

* Synge, Gordon, and Martin (154).
** Holiday (66).
† Corrected by deducting 25% of the ammonia present.

except that in order to "rationalize" this composition we must assume the extra water molecule to have hydrated some structure other than a peptide

bond in order that the number of amino and carboxyl groups be not more than are actually found.

A likely arrangement of the residues is a peptide chain in which the 19 α-amino and carboxyl groups are combined, presumably formed in a cycle. The 2 basic groups of the ornithine side chains would be free to combine as hydrochloride, while 3 of the 4 acidic side chains of the dicarboxylic acids are probably combined with ammonia as —CO—NH$_2$ groups. The remaining free carboxylic side chain presumably accounts for the free weak acid group found when tyrocidine is titrated by alkali in alcoholic solution. Of course, it is also possible that this group is combined and one of the α-amino acid carboxyl groups is free. It had been suggested that the free weak acid group was a phenolic group of tyrosine because the acetyl derivative appeared to be neutral (70), but this observation may have been in error.

The recent work of Gordon, Martin, and Synge has provided us not only with an approximately complete analysis of the constituents of tyrocidine, but also evidence of their optical configuration (49). Phenylalanine was recovered largely as the d-isomer, while the other amino acids were chiefly in the l-form. The d-phenylalanine accounts for 13% of the total nitrogen (calculated, 11.5%), while about 15% of the nitrogen was oxidizable with d-amino acid oxidase, none of it being in the dicarboxylic amino acid fraction (90). The simultaneous incorporation of d-leucine into gramicidin and l-leucine into tyrocidine by *B. brevis* is an interesting example of specificity in synthesis, as already noted (49). d-Phenylalanine and l-ornithine apparently had not previously been recovered from proteins that were not drastically treated. Gordon, *et al.*, discuss the possibilities that ornithine is a secondary product formed during hydrolysis of tyrocidine or autolysis of the *B. brevis* cultures, concluding that it is present in the tyrocidine as isolated. These authors bring support with model hydrolyzates to the earlier suggestion that hydrolysis with exclusion of air diminishes loss of tryptophane (70), also finding carbon dioxide production cut down. In spite of this precaution, the destruction of tryptophane was very extensive during their hydrolysis of tyrocidine, making it for the moment impossible for them to estimate the quantity or to determine the configuration of this amino acid.

The determination of tryptophane by Holiday (66) was by a spectrometric method (64, 65). The tyrosine content was placed by the same method in the vicinity of 7–9% of the total nitrogen.

The assumptions and approximations which have been used in making the calculations for tyrocidine, and for gramicidin, are no more than one is

quite at liberty to use in considering smaller organic molecules. Until their use is rendered unnecessary by the availability of the more precise analyses required for these larger molecules, they can be said to have helped reveal the broader structural outlines. For tyrocidine in particular, we have been able to formulate a fairly satisfying picture of the composition and general nature of a rather complex polypeptide structure. The apparent repetition of all its relatively complex amino acid residues within short compass is interesting to contemplate in relation to the structure of the higher proteins. The possibility that gramicidin and tyrocidine are large cyclic molecules remains at present entirely hypothetical.

4. Action of Enzymes on the Antibacterial Substances

The soluble protein precursor of tyrothricin, when acted upon by pepsin, crude trypsin, crystalline trypsin, or chymotrypsin, did not lose its biological activity (29). Instead, an active, insoluble form, presumably identical with tyrothricin, was caused to precipitate. Tyrothricin, treated with trypsin, papain, erepsin, and "bacterial protease," precipitates but is not digested (68). Similar attempts were made to hydrolyze partially purified gramicidin and tyrocidine. by crude trypsin, pepsin, papain, and papaya latex at several pH values. No increase in free amino or carboxyl groups was detected (70).

The resistance of these polypeptides to enzymatic hydrolysis is very likely attributable to the content of d-amino acids (90) and perhaps also in part to the large numbers of hydrophobic side chains. A suitable microbial, mold, or other enzyme preparation able to hydrolyze these substances can probably be found; it should reveal new interesting details of their molecular structure.

IV. Biological Properties of the Antibacterial Substances from *Bacillus brevis*

1. Effect of the Substances on Bacteria

In the early studies on spore-forming bacteria already mentioned, the manifestations of antagonistic action noted were, commonly, growth inhibition (bacteriostasis) or killing (bactericidal action) and in many cases actual dissolution (bacteriolysis) of the susceptible organisms. Tyrothricin in amounts of 0.01 µg./ml. and less is bacteriostatic for several species of Gram-positive cocci, while 1 µg./ml. or more is bactericidal and, in the case of such organisms as pneumococcus and certain others, induces bacteriolysis.

TABLE V

EFFECTS OF GRAMICIDIN, TYROCIDINE, AND TYROTHRICIN ON VARIOUS BACTERIA*

Microorganism	Gramicidin		Tyrocidine		Tyrothricin		Ref. No.
	Water	Peptone	Water	Peptone	Water	Peptone	
1. Gram-Positive Bacteria							
Pneumococcus	0.01—	0.5—	1	40	0.01—	10	28
		30, 100, 3		2, 5, 3		2, 10, 3	132
		1		20		1	55
						2, 0.006	32
						2, 0.7	127
						0.5	68
Streptococcus hemolyticus	2						28
		10, 10—	5	10, 10		20, 1	63
		40, 1		10, 3		10	132
		5		100			55
		0.002		0.2		0.01	125
						0.2, 0.2	126
						2, 0.6	127
						2	32
						1	148
S. hemolyticus, Group D		0.2				1	144
Streptococcus fecalis		40					33
				300		20	55
						30, 5	127
						10, 1	135
						5, 1	135
Streptococcus salivarius	1	2	8	15			34
Staphylococcus aureus	10	150, 150	50	25, 150, 100		20, 150, 125	132
							28
		300		300			55
		5–12					118
		20					101
							32
Micrococcus lysodeikticus	10	0.08	3	1		10	126
						6–800, 6–200	127
						180, 80	148
						25	144
Micrococcus conglomeratus		4		7		1	163
Sarcina lutea		5		3		0.1	156
						5, 10	163

(Table continued from preceding page; column headings appear on the previous page. Italicized figures = bacteriostatic, roman figures = bactericidal.)

Organism							Ref.
Gaffkya tetragena			*0.6*				101
Lactobacillus acidophilus							166
Corynebacterium diphtheriae						3	144
Clostridium welchii			*100,2*	10,10	*40,60,10*	1	132
					0.1–1		102
Clostridium tetani			*150*	2	*2*	3	132
Bacillus subtilis	1000+		*300+*	300+	*2*	3	163
Bacillus mycoides	1000		*300+*	300+	*3*	10	163

2. Gram-Negative Cocci

Organism							Ref.
Neisseria meningitidis	5		*40*	1	5	*50+*	28
						10	144
Neisseria gonorrhoeae	1		*20*	0.01–	0.1	*10*	28
Neisseria catarrhalis			*40*			*600*	148

3. Other Gram-Negative Bacteria

Organism							Ref.
Escherichia coli	200+		*200+*	15	200+		34
	500+			500			28
			300+	1000+	*300+*	*300+*	163
			800+		*800+*	*800+*	132
						800	148
Eberthella typhi	500+		*800+*	500	*800*	*800*	132
				1000+			148
							28
Aerobacter aerogenes	500+		*300+*	500	300+	*300+*	163
Shigella dysenteriae	500+		*800+*	1000+	300+		28
Pasteurella tularensis	250			50			28
Salmonella schottmuelleri	500+			100			28
Salmonella paratyphi				250		*800*	148
Brucella abortus			*100*				163

4. Other Organisms, Fungi

Organism							Ref.
Actinomyces spp.	300+		*300*	3	10	*30+*	163
Achorion schoenleinii						*50*	148
Microsporium gypseum						*100*	148
Trichophyton gypseum						*100*	148
Candida albicans						*500*	148

* Figures given are the minimum number of micrograms per ml. of suspension required to show a bactericidal (roman type) or a bacteriostatic (italicized type) effect. "Water" refers to suspensions of bacteria in water, buffer, glucose, or synthetic media. "Peptone" includes suspensions in peptone, broth, serum, and nutrient agar media.

As the material was fractionated with organic solvents, however, it was found that bacteriolytic activity was not always parallel with killing power. This observation became useful as a guide in making separations; and when isolated constituents were available, the explanation became obvious. Pure gramicidin can be bacteriostatic (and bactericidal to some degree) for Gram-positive cocci at levels of 0.01 to 1 μg./ml. (1:100,000,000 to 1:1,000,000); but very much larger amounts do not induce lysis of any organisms. Tyrocidine hydrochloride, on the other hand, kills in concentrations usually 10 to 50 times larger; and bactericidal action is attended by bacteriolysis of those organisms capable of rapid autolysis (34). Separation of pure constituents has therefore sharpened our judgment by requiring us to define the criteria by which the activity of antibacterial extracts is judged.

A compilation of data from the literature is presented in Table V to record observed activities of gramicidin, tyrocidine, and tyrothricin against a variety of bacteria. The data demonstrate the principal points which are:

1. Generally speaking, all three agents are more active against Gram-positive organisms than Gram-negative ones.

2. Exceptions to (1) are that the *Neisserieae* respond more like the Gram-positive cocci and that the Gram-positive, spore-forming bacilli are insensitive to gramicidin.

3. Tyrocidine has some activity against Gram-negative organisms.

4. Gramicidin is primarily bacteriostatic.

The data in Table V illustrate the wide range of variation to be expected in results from different laboratories, when strains of bacteria, methods of testing, and media are different. A bactericidal test involving short exposure to poison, prolonged incubation of a subculture (28), or reading of mere presence or absence of growth without reference to number of surviving cells (as was done in virtually all the studies quoted) is likely to be a severe test, since survival of an altogether negligible number of individual organisms (or spores) can be judged as complete growth, and therefore resistance. Where the number of surviving cells was determined by count (63, 132, 156), a more reliable index can be expected. In some cases, strains of staphylococci of greater than average resistance were consciously used (126, 132). Where bacteriostasis is measured, a count of surviving cells (55, 63, 132) is highly desirable, and the composition of the medium has a great effect upon the result. At moderate concentrations, gramicidin and tyrocidine are flocculated by the electrolytes in most media; this appears to have little effect upon their activity, although it may mean that the actual concentration of agent is not that calculated. Perhaps because of the diminished diffusibility and dissolution away from precipitated material, these agents are somewhat less effective in agar cultures than in liquid media (163). Some of the data in Table V were obtained in agar media (148, some in references 28 and 163). In the presence of protein, as will be noted later, the precipitation of tyrocidine materially reduces the effectiveness of this agent. A tech-

nique used by Herrell and Heilman (55) measures by colony count the bacteriostatic effect in the presence of growing tissue cultures, and appears to be an *in vitro* method which more closely imitates *in vivo* conditions than the usual methods.

Another determining factor in the bacteriostatic tests is the duration of the experiment. In many cases the bacteriostasis *in vitro* is short-lived for the reason that resistant organisms can develop and eventually give full growth. This phenomenon has frequently been noted with gramicidin (33, 35, 72, 118) and tyrothricin (118, 126), especially with staphylococci. The nature of the resistance developed is obscure. The fermentation reactions do not appear to be changed (118), but the modified strains are often very slow-growing even in normal media (72, 118). Robinson and Graessle have suggested that, in blood cultures, late growth developing after bacteria have been inhibited for a period by gramicidin may be the result of phagocyte destruction by the agent (132).

Neter has found 2 μg./ml. of tyrothricin to inhibit streptococcal fibrinolysis and plasma coagulation by staphylococcus cultures; there was some question whether the lysin and coagulase were themselves so affected. In a later article Neter described the similar action of a cationic surface-active agent (111). The same worker has reported tyrothricin to delay the spontaneous inactivation of diphtheria and tetanus toxins (112). Staphylococcal toxin is not affected by tyrothricin, although neutralization of the hemotoxin by antitoxin was inhibited (10).

Added to ordinary media, tyrothricin in concentrations sufficient to inhibit growth of Gram-positive bacteria aids in the isolation of *H. influenzae* (145) and *N. gonorrhoeae* (151). Gramicidin-containing media have been used similarly for isolating various Gram-negative organisms (33).

2. Natural Inhibitors of the Action on Bacteria

Results obtained with tyrocidine in bactericidal tests are considerably influenced by the presence of protein and protein split products such as peptones and proteoses. Probably this can be attributed to the ability of this basic polypeptide to combine, and in some cases precipitate, much as protamines do, with protein derivatives. The more inert, neutral gramicidin does not appear to have this property, and its action is not greatly affected by protein derivatives. A notable demonstration of such inhibition is the following: Tyrocidine (15 μg./ml.) in buffer or saline suspension is bactericidal and bacteriolytic for Gram-negative as well as Gram-positive bacteria, but there is no action by even 200 μg./ml. when the test is made in peptone media. This observation is true of representatives of the Gram-negative groups, *Escherichia, Klebsiella, Shigella, Salmonella, Hemophilus,* and *Neisseria* (34). The action on Gram-positive organisms is likewise considerably decreased by peptone. These effects are not clearly shown in Table V, since few of the data with and without peptone were obtained under comparable conditions.

The inhibition of tyrocidine is more striking still when media containing proteins are used (Table VI).

The action of gramicidin is little affected by peptones, pure proteins, carbohydrates, sterols, fatty acids, etc., so far as is known, but there is a mild degree of inhibition by such agents as serum. This appears to be due principally to the cephalins (33, 35), both phosphatidyl serine, the form recently identified by Folch (40), and phosphatidyl ethanolamine, the classical cephalin. The quantity of these phosphatides present in serum, ordinary media, etc., is not great enough to have much effect; but when purified phosphatidyl serine is added to bacteria *in vitro* or *in vivo*, it is able to inhibit the action of as much as one-half its weight of gramicidin, added

TABLE VI

EFFECT OF AGENTS ON BACTERIA IN PLAIN AND PROTEIN-CONTAINING MEDIA*

Bacterium	Gramicidin		Tyrocidine		Tyrothricin		Note
	Plain	Protein	Plain	Protein	Plain	Protein	
Streptococcus hemolyticus	64	128	2	128	4	64	1
Streptococcus hemolyticus	1	32	1	64	1	32	2
Pneumococcus Type I	0.5 −	0.5 −	1	40	....	..	3
Meningococcus	10	40	10	40	....	..	3
Pneumococcus Type I	....	.....	..	...	0.01 −	1 −	4
Meningococcus	....	....	..	...	0.01 −	50	4

* Figures are the lowest number of μg./ml. able to show effect.
NOTE 1: Bactericidal test (132). Plain medium was brain-heart infusion; protein medium was blood. Staphylococcus behaved similarly.
NOTE 2: Bacteriostasis, 4 hours (132). Otherwise same as note 1.
NOTE 3: Bacteriostasis, 13 hours (28). Plain medium was proteose–glucose agar; protein medium was proteose–hemoglobin agar.
NOTE 4: Bactericidal test (28). Plain medium was 5% glucose; protein medium was 4.5% mucin.

afterwards (33). The basis for this inhibition has not been discovered; it should be pointed out that other cases of inhibition of germicides by phosphatides have been reported (13, 159, 169 and review in 4). Baker, Harrison, and Miller have found cephalin, sphingomyelin, and lecithin to inhibit the depression of bacterial metabolism caused by tyrothricin and also the metabolic and bactericidal effects of synthetic detergents (4). Here, also, to be effective the phosphatide had to be added first.

There can be obtained from Gram-negative bacilli a protein-free fraction rich in phospholipids which can also inhibit gramicidin (33, 35). This may be a general finding, since the material appears to be a part of the antigenic complex typical of Gram-negative bacteria (11, 106). Whether this fact

can account for the resistance of this class of organisms to gramicidin it is not possible to say, but it is quite conceivable that it does account for the greater resistance of Gram-positive organisms when Gram-negative bacteria are also present (35). Miller, Abrams, Dorfman, and Klein have reported that treatment with protamine, which may remove phospholipids, renders Gram-negative bacteria much more susceptible to tyrothricin than they normally are (103).

Dubos did not find the bacteriolytic or bactericidal effects of the water-soluble "proteinaceous" agent, from which tyrothricin is presumably derived, to be prevented by the presence of meat infusion, peptone, serum, or ascitic fluid (29, 30).

TABLE VII

SUMMARY OF EFFECT OF AGENTS UPON BACTERIA IN DIFFERENT MEDIA

Agent	Buffer suspensions		Serum, peptone, or *in vivo*	
	Gram-positive	Gram-negative	Gram-positive	Gram-negative
Gramicidin	Bacteriostatic	Slight	Bacteriostatic	Slight
Tyrocidine	Bactericidal	Bactericidal	Slight	Slight
Tyrothricin	Bactericidal	Bactericidal	Bacteriostatic	Slight

There is ample evidence that tyrothricin contains substances other than gramicidin and tyrocidine; and it is almost certain that some of them have antibacterial activity. Nevertheless, in saline, buffer, or glucose suspensions, tyrothricin behaves much as a mixture of the two pure substances would be expected to behave. That is to say, it appears to be essentially a mixture of "gramicidin-like" and "tyrocidine-like" components, even though neither every one of these active components, nor all of the activity, has been isolated in crystalline form. In peptone- or protein-containing media, on the other hand, tyrothricin has principally the action of its gramicidin-like components, since tyrocidine here is largely inhibited from acting. Table VII summarizes this state of affairs.

3. Effect of the Agents on Bacterial Infections

The marked effectiveness of the products of *B. brevis* in repressing bacterial growth in the test tube naturally raised the possibility that they could be used to combat bacterial infections in the animal. In addition, the relatively high specificity of their action for certain microorganisms led to the hope that they would be selective enough not to injure the tissues of the host. The first experiments were striking, and indeed promising. Mice

could be protected against an invariably fatal intraperitoneal infection with various bacteria by the intraperitoneal administration of minute doses of the substances. Thus, mice survived 10,000 times the minimal fatal dose of Type I pneumococci if they also received either 2 μg. of gramicidin (34, 74), 50–100 μg. of tyrocidine (34, 74), 5 μg. of tyrothricin (32), or 2 mg. of the protein-containing agent (30). Other susceptible organisms can with equal success be prevented from multiplying in the animal in similar experiments. However, administration of the agents subcutaneously, intravenously, or intramuscularly had no therapeutic effect, except that irregular protection was sometimes obtained by subcutaneous use of the protein fraction (31, 101). The treatment, to be successful, had not only to be applied directly to the same site as the infection, but was chiefly *protective*, being *curative* only if the infection had gained a foothold during no more than a few hours (30, 32, 101, 132).

This experimental infection can be considered as a model of the conditions under which gramicidin and tyrothricin can be protective or curative *in vivo*. They have found their chief application in analogous localized infections of body cavities, wounds, or ulcers. Tyrothricin, more available than gramicidin, is the form generally employed, often being wrongly designated as gramicidin. Tyrocidine is generally considered ineffective *in vivo* and is little used in pure form. Not all infections proceed as rapidly from a localized (treatable) to a septicemic (and untreatable) phase, as does the pneumococcus intraperitoneal infection of the mouse. However, the existence of inaccessible foci, or fibrin barriers, and the presence of inhibitors are variables which make almost every infection a special case.

Complete protection by gramicidin or tyrothricin was achieved in mice when the organism infecting the peritoneal cavity was the pneumococcus of Types I, II, III, V, and VIII, or streptococcus—11 of Group A and 5 of Group C—(31, 32, 35, 74), sulfonamide-resistant pneumococcus (155), or *B. anthracis* (68, 101). Prolongation of life occurred when staphylococcus or *Clostridium welchii* in mucin were given (132). It is probable that mucin, added to increase mortality, serves not only as a handicap for the natural defenses of the animal, but also for the action of the agent; this appeared to be true both *in vivo* and *in vitro* when meningococci, chiefly affected by tyrocidine, were used (28). The small quantities (up to 2 μg.) of tyrothricin injected intramuscularly into mice in an attempt to control *Cl. welchii*, although enough in the test tube (102), should hardly have been expected to protect the animals, even if the route were more advantageous. Protection by tyrocidine is usually much less than by gramicidin (34): it requires more prompt injection (19), and is not successful against Gram-negative organisms which succumb *in vitro* in the absence of peptone (34).

In the pleural cavity of the rabbit, 3–40 mg. o tyrothricin could sterilize hemolytic streptococcus empyema, but staphylococcic infections were more resistant (128).

Similar infections in man responded only rarely, staphylococci and also pneumococci being little affected in a short series of cases (127).

Rammelkamp (127), Herrell and Heilman (54, 55, 56), Wright (170), and Bordley, Crowe, Dolowitz, and Pickrell (12) have summarized their clinical experiences with tyrothricin. Infection was controlled in ulcers carrying streptococci and frequently staphylococci (55, 127, 170). Postoperative treatment of the mastoid cavity eliminated streptococci, staphylococci usually appearing in their place (12, 127), and led to greater comfort of the patient and shorter hospitalization (12). Streptococcal sinusitis was cleared up, but not the staphylococcal form (55, 127). Osteomyelitis was sometimes benefited, but a critical factor was having the agent come in contact with the infecting organism (127, 170). Various superficial infections were treated with tyrothricin with irregular success (55, 127), while Wright reports favorable results with these and a variety of other miscellaneous infections (170). Herrell and Heilman summarized the results with 50 cases of all types as: good, 43%; fair or temporary improvement, only 25%; and poor 32% (56). In reviewing the extension of this series, Herrell states that 75% of 150 cases showed fairly good to excellent response (54). The series included pre- and postoperative wounds, infected ulcers, sinus and bladder infections, and a few cases of empyema; it is to be supposed that the experience of these workers has enabled them to choose cases in which tyrothricin would presumably be of value. Pulvertaft states that treatment of war wounds with tyrothricin and other agents nearly always caused the appearance of secondary invaders as the Gram-positive organism disappeared (124). Several observers have suggested that, when an infection was treated, the healing process itself may have been promoted by tyrothricin (12, 55, 130, 170).

Schoenbach, Enders, and Mueller have reported elimination of hemolytic streptococci from the nasopharynx of monkeys and human carriers by spraying with tyrothricin (144). Francis was successful in sterilizing one of two cases of wounds infected with sulfonamide-resistant streptococci (42). Robson and Scott were unable to affect staphylococcal eye lesions in rabbits; they applied tyrothricin as a saline suspension of the powder, without benefit of previous alcohol solution to subdivide it (134). Bellows reports that results in acute conjunctivitis were unimpressive (7). Knee joints of rabbits were sterilized by tyrothricin when infected with streptococci, but not when they carried staphylococci (128). Pico states that gramicidin (tyrothricin?) has some protective action in guinea pigs infected with diphtheria bacilli (121).

Rammelkamp noted the development of resistance in a strain of staphylococcus during treatment of an ulcer patient (126). He has summarized the factors determining success of therapy with tyrothricin, and concludes that the natural resistance of the infecting organism, development of resistance especially by staphylococci during therapy, accessibility to direct contact, mechanical carrying away by exudate, and presence of inhibitors are the important factors (127). Among the inhibitors he includes the Gram-negative organisms, which are not only themselves resistant but in mixed infections protect the otherwise susceptible Gram-positive organisms. The possible relationship of this effect to cephalin inhibition has been mentioned above.

Tyrothricin has been fed to animals in attempts to modify the intestinal flora, with varying success. The tyrocidine component was found not to affect in the intestine the Gram-negative organisms that were susceptible in peptone- and protein-free media (34, 135). Neither was orally administered tyrothricin effective against *Lactobacillus acidophilus* (166). This last experiment was probably a demonstration of the inhibition of the gramicidin component by feces (127), possibly by the Gram-negative organisms

in the mixed population. Such a conclusion appears to be supported by the finding of Rodaniche and Palmer that it was difficult to observe repression of the fecal streptococci in the intestinal flora unless sulfasuxidine, which inhibited the Gram-negative organisms but not the streptococci, was given along with tyrothricin (135).

Little, Dubos, and Hotchkiss employed tyrothricin and partially purified gramicidin in treating streptococcic mastitis of cows. Injection into the infected udder, another essentially "local" use of tyrothricin, was considered successful by the authors in some 65% of the treated quarters (92). Numerous later investigations in this field (1, 6, 14, 15, 37, 38, 69, 91, 93, 99, 100, 109, 141, 143, 157, 158) have been in general agreement with the optimistic view that permanent cures can be obtained by this means in a considerable majority of cases, with a minimum of damage to the secretory tissue.

4. Toxicity of the Agents for Animal Cells and Tissues

The complex question of the toxicity and pharmacology of these bactericidal substances cannot be adequately treated here. However, some aspects of the problem bearing on *in vitro* mechanisms will be of interest to us, and enough will be indicated of the animal experiments to reveal the high order of parenteral toxicity of these substances.

Washed mammalian erythrocytes are hemolyzed by tyrocidine (50–100 μg./ml.). The lysis resembles the "physical" effects of saponin and detergents, occurring within a few minutes after mixing, except when only very small amounts of tyrocidine are used (34). Crude tyrothricin behaves similarly (51, 101), but gramicidin does not (34, 101), nor does tyrocidine in serum. Dubos and Hotchkiss (34) and Rammelkamp and Weinstein (129) ascribed the hemolytic properties of tyrothricin to its content of tyrocidine, although the latter workers found some hemolysis by two samples of gramicidin which appear to have contained some tyrocidine. It seems certain that the method of Dimick for determining tyrothricin by its hemolytic action over short intervals (26) essentially measures the tyrocidine-like components. Herrell and Heilman, on the other hand, pointed out that gramicidin has hemolytic properties, and regarded it as the principal hemolytic agent in tyrothricin (55). This it may possibly be, when minimal quantities of tyrothricin are used in blood or serum (97). Gramicidin does not produce rapid, extensive hemolysis like tyrocidine, but rather a delayed lysis beginning only after several hours and continuing progressively for many hours. The striking thing about hemolysis by gramicidin is the small amount of the toxin (0.5–1 μg./ml.) needed to elicit it. This action was blocked by the glucose in the suspensions first used (34), although the minimum concentration of glucose (2 mg./ml.) preventing hemolysis by gramicidin is above the physiological range (56) and this sugar has no effect upon hemolysis by tyrocidine (34, 129). Carbon compounds other than glucose can interfere with hemolysis by gramicidin (71), and cephalin inhibits the hemolytic, as it does the bacteriostatic, action (35). Nevertheless, progressive hemolysis of the gramicidin-induced type can occur in the presence of tissue culture clots in serum—partial at 20 μg. (55) or complete at 0.5 μg. (58) of gramicidin per ml.—and probably also under *in vivo* conditions. This possibility would go a long way toward explaining the hemolytic effects and anemia observed after parenteral administration of tyrothricin, according to Herrell and Heilman (56). It seems very likely that tyrocidine as well, in the local high concentrations

resulting after intravenous or parenteral injection, can be expected to overcome serum inhibition sufficiently to produce intensive red cell destruction.

In tissue cultures, microscopic examination did not reveal any damage to leucocytes by 100 μg. of gramicidin or tyrocidine per ml. of blood (55). Under other conditions, tyrocidine in quantities of 500 (35), 160 (130), and 25 μg./ml. (20) was said to be leucocytolytic, even in serum, and inhibitory to phagocytic activity (20, 130). Tyrothricin was similarly toxic, but gramicidin in quantities of 500 (35) and over 2000 μg./ml. (130) did not have these effects. As was the case with hemolytic activity, however, studies with a more delicate technique enabled Herrell and Heilman to show that gramicidin was more toxic than tyrocidine, and to conclude that the cytotoxicity of tyrothricin was largely due to gramicidin. The migration of lymphocytes from lymph node explants in serum-containing tissue cultures was decreased in extent by 100 μg./ml. of gramicidin while the same amount of tyrocidine had no effect (59). Similarly, macrophage migration from spleen fragments was considerably cut down by 10 μg./ml. of gramicidin or tyrothricin, while 100 μg./ml. of tyrocidine had a smaller effect (58). These observations represent only a mild degree of toxicity compared with many germicides or the hemolytic activity of these same agents (58).

Among other observations concerning cytotoxicity may be mentioned the lysis of protozoa by tyrocidine (71), the destruction of motility of spermatozoa by 10 μg./ml. of gramicidin and tyrothricin but not tyrocidine (53) and the relative inertness of all the preparations toward tissue cultures (55, 122).

Their acute toxicity toward animals places these fractions among the highly active poisons. Intravenously, quantities of about 2 mg./kg./day of gramicidin or tyrothricin kill rabbits or dogs (19, 101, 133), while 10 mg. of tyrocidine (19) or 0.4 mg. of purified tyrothricin* (95) per kg. per day killed dogs within a few days. For mice, the toxic intravenous dose was placed at 0.8 mg. of tyrothricin and 6 mg. of gramicidin per kg. per day (101), while, in a careful titration, Robinson and Molitor had 50% deaths at single doses of 3.7 mg. of tyrothricin, 15 mg. of tyrocidine, or 2.5 mg. of gramicidin, per kg. (133). These last investigators reported 50% mortality on intraperitoneal injection of single doses of 20 mg. of tyrothricin or 40 mg. of tyrocidine or gramicidin per kg. into mice. Other scattered observations are in essential agreement with these last figures (34, 74, 101, 130). All workers agree that, given orally, these substances have virtually no toxic effect (34, 101, 135, 166, 170), even in quantities as great as 1000 mg./kg. (133).

The minimal lethal doses mentioned above are not the minimal toxic doses. After giving smaller doses (0.3 mg. purified tyrothricin/kg./day) intravenously to dogs, MacLeod, Mirick, and Curnen noted ascites and, on histopathological examination, fatty degeneration of the liver (95); but the effects of 0.2 mg. were minor. Other effects of small doses were loss of weight with anorexia, loss of muscle tone, rise in temperature, and salivation shortly after injection (95, 133). Lethal amounts of tyrothricin gave a general picture of restlessness, loss of weight, anorexia, depression, progressive anemia, leucocytosis, ascites, hematuria, excretion of bile in the urine, followed by fall in temperature, respiratory failure, and death (95, 133). The experiences with pure gramicidin were closely similar except that death occurred sooner and anemia and leucocytosis were not noted before this happened (133). At necropsy, the abnormal findings in-

---

* This preparation was tyrothricin from which some tyrocidine had been removed. It probably contained on the order of 40% gramicidin, 20% tyrocidine, and 40% other fractions.

cluded acute congestion of the lungs and abdominal viscera, petechial hemorrhages of lungs and kidneys and the myocardium of the heart, and diffuse hemorrhage of the spleen. On histopathological examination, fatty degeneration and central necrosis of the liver, cloudy swelling of the tubular epithelium, and hemorrhage in the glomeruli of the kidneys were found (95, 130, 133). As Robinson and Molitor point out, these are more or less nonspecific degenerative changes (133).

In anesthetized animals, tyrocidine depressed the respiration somewhat, while similar small doses of gramicidin did not (133). When aspirated from the nasal passages, or injected into the trachea, 5 mg. of tyrothricin produced consolidation in the lung of rabbits, and 10 mg. sometimes had a fatal effect (130).

At various local sites, toxicity was somewhat less pronounced. In the pleural cavity, tyrothricin caused only slight thickening of the pleura when less than 20 mg. was administered to rabbits. As much as 200 mg. has been given by this route to human patients without toxic reactions, although there may have been protection by a fibrin barrier in these infected cases (130). In the knee joints of rabbits, less than 2 mg. of tyrothricin had little effect; more produced some induration with fibrosis and leucocytic infiltration (130). Similar effects are observed in the skin after intradermal or subcutaneous injection (42, 130), 10 μg. of tyrothricin producing at least as marked induration as 200 μg. of gramicidin (130). The nodules formed persist for several weeks (133). Dry preparations of gramicidin and tyrothricin were irritating to the eye, but 0.5 and 1 mg./ml. saline suspensions were introduced into the conjunctival sac without effect (7, 133, 134). Rectally (101, 170), tyrothricin had no toxic effects. Considerable amounts can be tolerated intramuscularly (101).

The lower toxicity of tyrothricin in local applications is, in general, confirmed in the clinical experience with this material (12, 56, 127, 170). Application in the form of alcoholic solutions to the site of recent wounds or operation has, however, sometimes caused hemolysis and fresh bleeding (130).

The effect of slow diffusibility in agar to delay and localize hemolysis by tyrothricin has been noted (51); the similar effect on bacteriostasis was mentioned above. Such mechanical factors as localization, precipitation, and diffusion presumably are of great moment in determining where and in what tissue the demonstrated cytotoxicity of the agent will be manifested when it is given to animals.

The animal experiments just summarized have not brought out clear-cut differences between the toxic properties of gramicidin and tyrocidine, partly because most of the studies were conducted with the mixture, tyrothricin. There is, however, some basis for the expectation that their *in vivo* properties may in some respects be qualitatively different. Frequent mention has been made of the inhibition of the action of tyrocidine by proteins, peptones, serum, etc. The explanation suggested was that tyrocidine, having a hydrophobic-cationic nature, like the cationic detergents, precipitates or combines with protein anions. One might conclude that protein matter would prevent tyrocidine from ever having an action on tissue. However, if the protein with which tyrocidine combines is a protein vital to the cell or tissue and not merely part of the environment like serum protein, then combination would be expected to result not in

inhibition but in major injury to the tissue, probably very different from whatever effect gramicidin might have.

That tyrocidine can indeed have appreciable toxic activity when injected into the blood stream is evident from its effect on respiration of anaesthetized animals and its lethal intravenous action already mentioned.   It is naturally more easily observed in the absence of serum.   The various cytolytic effects discussed above may be interpreted as examples of tyrocidine acting upon cell constituents rather than constituents
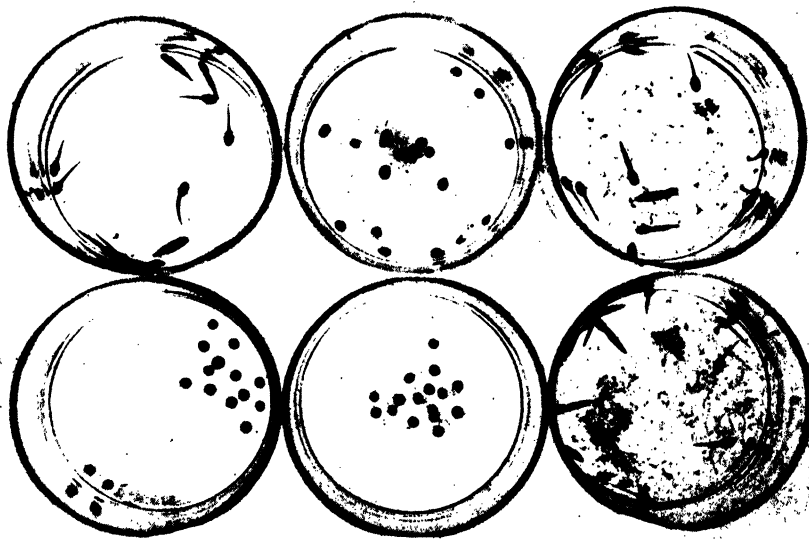
Fig. 4.—Frog eggs after six days' exposure to: (1) dilute alcohol control;   (2) tyrothricin, 63 µg./ml.;   (3) tyrocidine 6.5 µg./ml.;   (4) tyrocidine 38 µg./ml.;   (5) gramicidin 63 µg./ml.;   (6) gramicidin 310 µg./ml.

of the medium.   The experiments of Robinson and Molitor on isolated organs (133) may be of this type.   Isolated rabbit intestine in Locke's solution responds with marked contraction to 64 µg. of tyrocidine and not to 256 µg. gramicidin, per ml.   Similarly, 32 µg./ml. of tyrocidine decreases and soon terminates the beat of the isolated frog heart preparation in Ringer solution, while 264 µg. of gramicidin has no effect or only a slight one.

A similar conclusion is suggested in an unpublished experiment with frog's eggs.   Fertilized eggs from a single batch were placed at an early blastula stage in Ringer solution, together with various amounts of gramicidin, tyrocidine, and tyrothricin.   At two days,

all embryos exposed to tyrocidine or tyrothricin had white patches of dead cells on the vitelline membrane and were apparently dead, while those in gramicidin appeared to be like the controls.* The appearance after six days at 25° C. is as reproduced in Fig. 4. Note that, in vessel (2), the embryos were exposed to 63 μg. of tyrothricin per ml.; the approximate concentration of the two polypeptides can therefore be estimated as 15 μg. of gramicidin and 40 μg. of tyrocidine per ml. Obviously tyrocidine can account for the total observed toxic effects, while gramicidin in large amounts allowed the development of normal-appearing tadpoles. The gramicidin was partly, though not totally, precipitated, so its insolubility may be one of the inherent properties which permits a low local toxicity. The albuminous jelly surrounding each egg became clouded in high concentrations of tyrocidine, but did not prevent small amounts of this agent from killing the embryo.

Robinson and Molitor have suggested that the somewhat greater solubility of tyrocidine in water is accountable for its greater action than gramicidin in some situations (133). The suggestion made above that, once in solution, the demonstrated protein-combining power of tyrocidine can serve as an explanation of tissue toxicity is little more than assumption. Of course, there may also be a combination with anions such as nucleic acids, phosphatides, acid polysaccharides, etc.; or entirely different principles may be involved. Whatever the mechanism, some inherent property of tyrocidine enables it to act upon intact tissue under certain circumstances, and to have definite toxic effects. These effects have been suspected to be serious enough that it has several times been suggested that the therapeutic properties of gramicidin or an easily prepared acetone extract of tyrothricin might be more promising than those of the more available mixture, tyrothricin. That a very considerable residue of toxicity would remain in the pure gramicidin is unquestioned.

V.   Mode of Action of Gramicidin and Tyrocidine

1.   Structure in Relation to Biological Activity

The chemical data presented above have given at least a general over-all picture of what types of molecule gramicidin and tyrocidine can be, and have tremendously reduced the number of exotic structures that can be expected. There is still little, however, that would cause one to predict the highly developed toxicity these substances have for certain cells. Gramicidin does appear to contain a unique aminohydroxy constituent, but this in itself is not an explanation of toxicity, for the hydrolyzate in which we

---

* In a passing mention of these experiments (35), the concentration of tyrocidine lethal for frog's eggs was incorrectly given as 10,000 times the actual one.

detect this constituent is inert.* No very unique constituent has been detected in tyrocidine. Both gramicidin and tyrocidine carry a considerable proportion of *d*-amino acids. These may be toxic when combined, or the peptide containing them may "compete" with some normal protein or peptide. However, it is more likely that the unnatural amino acids contribute rather a measure of biological indestructibility to a molecule toxic for other reasons, perhaps on account of some special mode of combination of an aminohydroxy constituent, or because of an abnormal content of indole or aliphatic side chains. As simple tests of such structural influence, substances like palmityl tryptophane have been prepared (71). As might be expected, palmityl-*l*-tryptophane has a slight bacteriostatic and detergent activity. The *dl*-compound, however, is no more active.

It appears to the reviewer somewhat more probable that the toxic property resides in larger elements of the structure than any single chemical group—in other words, that it is a "physical" property. Hotchkiss and Dubos pointed out the resemblance of tyrocidine to cationic detergents in its effect upon surface tension and also in precipitating proteins (77). Since many of the cationic detergents are bactericidal, this resemblance may be significant. Heilman and Herrell at the same time showed that gramicidin, too, has a smaller but definite, slowly established, surface-tension depressant action on aqueous solutions in which it is made sufficiently soluble (52). Because these workers found bactericidal and hemolytic properties to be lost, but surface activity to be retained, when gramicidin was heated they were inclined to relate its primary activity to a more specific property. However, "inactivation" by heating of the unstable aqueous suspensions of gramicidin or tyrothricin appears to be an unreliable guide, since it has been traced to practically invisible precipitation on the vessel walls, from which the material can again be recovered in active form if taken up in a mixture in which it is more soluble (35). Consequently, it will be wise to remember the suggestive observations made by Heilman and Herrell in considering the activity not only of tyrocidine but of gramicidin as well. Surface activity probably at least enables the substances to be effective in minute amounts, for it very likely results in their concentration and fixation at the surface of the bacterial cell. Of course, the most relevant measure of surface activity would be made at cell–water interfaces rather than at an air–water interface. In a recent article, Herrell and Heilman report that the action of gramicidin is inhibited by cationic surface-active agents such as Phemerol, and discuss again the surface

---

* Isoserine is reported to be inhibitory for the growth of yeast, entering into competition with pantothenic acid (114).

activity of gramicidin (57). The inhibition of a product, apparently tyro-
thricin, by phospholipids or a neutral detergent (4) may be a resultant of
this and other effects.

2. The Effects on Cellular Metabolism

While the chemical and physical data concerning gramicidin and tyro-
cidine have led to only very incomplete hypotheses concerning the basis
of their activities, it is felt that another side of the picture is even more in-
complete. Until more is known about the nature of the cell structures or
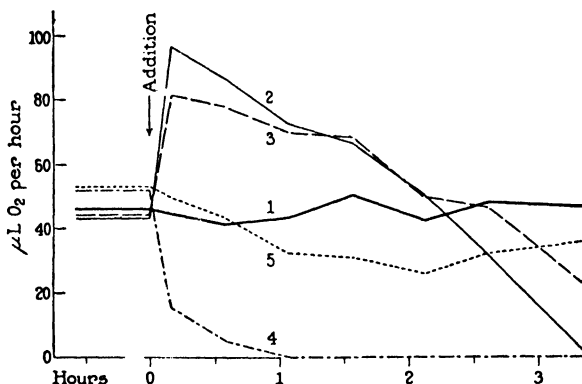
Fig. 5.—Rate of oxygen uptake by *Staphylococcus aureus* acting on glucose at 38° C.
The ordinate represents oxygen uptake per hour by 1.4 ml. of bacterial suspension con-
taining washed cells from 2 ml. of broth culture and 10 mg. glucose. (1) Control; (2)
40 μg. of gramicidin added at zero time; (3) 1 μg. gramicidin added; (4) 40 μg. tyrocidine
hydrochloride added; (5) 1 μg. tyrocidine hydrochloride added. MEDIUM: 0.07 M
potassium phosphate buffer at pH 7.4, containing Mg^{++}, Mn^{++}, Ca^{++}, and Na^+ in
addition to K^+. (Reproduced from *J. Biol. Chem.*, **146**, 421 (1942).

substances with which they interact, even these hypotheses cannot be made
more detailed or subjected to test. Accordingly, much of the remaining
space will be devoted to a discussion of recent chemical and metabolic
studies of the action of the pure polypeptides on suspensions of bacteria.

The soluble protein agent and tyrothricin were both shown to inhibit re-
duction of methylene blue by suspensions of susceptible organisms in glu-
cose (29, 32). The loss of reductase activity was quantitatively related to
the loss of viability, but which of the two effects might be causal for the
other was not clear. Dubos, Hotchkiss, and Coburn, taking up this prob-
lem, used the crystalline polypeptides in analogous experiments (36).

Tyrocidine added to staphylococcal suspensions acting on glucose produced an irreversible *depression* of the respiration (Fig. 5) even in small amounts. Gramicidin, on the other hand, even in very large amounts, brought out a pronounced *stimulation* of respiration maintained for over two hours.

While we have so far been able to deal with tyrocidine and gramicidin together in our discussions, their properties have often been in contrast. In what follows, and in all considerations of intimate biological mechanisms involving them, it is essential to treat the two substances separately. Their differences of action now necessarily become a major theme. The experiment represented in Figure 5 can serve as a pattern for all that can be said about the mechanism of action of these polypeptides. Tyrocidine has always been found to produce gross inhibitory and destructive changes, both on bacteria and other cells. The presence of gramicidin is, however, generally compatible with continued metabolic activity and life (*e. g.*, bacteriostasis), killing generally occurring slowly or not at all.

3. Mode of Action of Gramicidin

How, then, does gramicidin inhibit so markedly the growth of susceptible cells? First of all it is to be noted that Fig. 5 shows, not absence of effect, but indeed a very rapidly instituted and striking one. A similar stimulation of metabolism is also produced by gramicidin when staphylococci are oxidizing alcohol, and when Group D streptococci are converting glucose to lactic acid (36). But the action need not always appear as a stimulation. When potassium ions are withdrawn from, or ammonium ions added to, the medium in which staphylococci are suspended, gramicidin instead has no effect or else depresses the respiration (36). Magnesium and calcium ions heighten the stimulatory effect, and so does building up the reserve material of the cell by appropriate preconditioning (72).

There are reasons for supposing that effects of this kind are in some way connected with the influence on viability of the bacteria. They occur at as low concentrations of gramicidin, as do bacteriostatic effects. Equivalent responses of stimulation or partial inhibition are easily produced in other susceptible Gram-positive bacteria, such as *Sarcina lutea*, *Micrococcus lysodeikticus* and *Gaffkya tetragena*, but no influence is observed upon the nonsusceptible organisms such as yeast or *Escherichia coli* in similar experiments (72). Quite analogous effects on respiration and glycolysis of bovine spermatozoa were accompanied by loss of motility of these susceptible cells (53).

In the hope that one or some few metabolic reactions would prove to be the vulnerable loci at which gramicidin acts, attention has recently been

concentrated on the metabolism of *Staphylococcus aureus* as influenced by this agent (72). Staphylococci growing in stationary cultures convert glucose largely into lactic acid, small amounts of ethyl alcohol also being produced (45). However, under conditions of aeration, with the cultures used in this work, oxidation to acetic acid is the almost exclusive process, the over-all reaction being:

$$C_6H_{12}O_6 + 2O_2 \longrightarrow 2CH_3COOH + 2CO_2 + 2H_2O \ (R.Q. = 1.0)$$

Lactic acid and glycerol are oxidized to the same end products. Recent evidence that staphylococci produce phosphorylated intermediates (41, 152), adenosinetriphosphate (85, 94), etc., is good indication that they can carry out the conversions in accordance with the Embden-Meyerhof scheme.

TABLE VIII

METABOLISM OF SUSPENSIONS OF WASHED STAPHYLOCOCCI

Substrate	Theoretical R.Q.*	Without gramicidin		With gramicidin	
		Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
Glucose	1.0	74	0.96	146	0.96
Lactate	1.0	108	0.96	112	0.94
Glycerol	0.67	109	0.55	39	0.72
Glycerol (limited):					
first quarter	..	...	0.59	...	0.72
last quarter	..	...	1.02	...	0.70
over-all	0.67	...	0.72	...	0.71

* Theoretical R.Q. = respiratory quotient for oxidation to acetic acid plus carbon dioxide.

In the presence of gramicidin, the normal oxidation products are apparently produced in the usual proportions, only the rate of oxidation being changed. The oxidation of glycerol is considerably inhibited under the same conditions under which oxidation of glucose is doubled or tripled in rate. When lactic acid is the substrate, even the rate remains unaffected; this is taken to mean that the interference by gramicidin is at some point prior to the formation of pyruvic acid. The respiratory quotients characteristic of these normal and gramicidin-poisoned processes are given in Table VIII along with the rate of oxidation. The respiratory quotient of normal glycerol oxidation, it will be noted, is 0.55, materially below the theoretical (R.Q. = 0.67):

$$2C_3H_8O_3 + 3O_2 \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2O$$
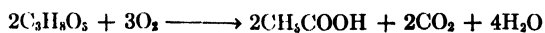
In the presence of gramicidin, however, the calculated value is obtained. This must mean that normally some oxidation product of glycerol accumulates which cannot do so in the presence of gramicidin. A test of this idea is obtained by supplying only a limited amount of glycerol, thus forcing the staphylococci eventually to oxidize the accumulated product in turn. The data indicate that the usual low value was found at first. When oxidation approached completion, however, the R.Q. rose sharply in the normal cell suspension so that, at the end, the over-all effect had been to complete the oxidation quantitatively to acetic acid. The gramicidin-treated cells were doing this steadily for the whole period.

Let us suppose that glycerol is oxidized to triose and then dissimilated, like hexose, according to the Embden-Meyerhof-Cori scheme. The temporary accumulation of any hexose or triose intermediate (R.Q. = 1.0) or phosphoglyceric, pyruvic acid, etc. (R.Q. = 2.0), would give the results observed with glycerol, *viz.*, a low initial and a high final R.Q. Where gramicidin prevented the accumulation of the intermediate, the theoretical R.Q. would be maintained. The final quotient of approximately 1 may suggest that the hypothetical intermediate is one of the carbohydrate derivatives; but it is only an average for all the processes occurring. To obtain a further clue, let us assume that the stimulatory effect when glucose is the substrate is due to this same inhibitory action of gramicidin which prevents production of the intermediate. Accumulation of a carbohydrate derivative would not give a low normal R.Q. here. It can be seen that stimulation of oxidation might then occur if the blocked process were a side reaction which normally diverted some glucose from the main pathway. If glucose oxidation proceeds by another pathway, *e. g.*, "end oxidation" (25, 87, 164), the arguments would be much the same. Since the data are not extensive enough to warrant further speculation, let us merely point out that:

1. continued accumulation of a phosphorylated intermediate would eventually be expensive in terms of phosphate,

2. there is a considerable amount of evidence, partly indirect, to indicate storage of a polysaccharide while substrates are being oxidized by normal cells (reviewed in 120, also 84, 167),

3. inhibition of polysaccharide synthesis by gramicidin would plausibly enough affect bacterial growth and, at the same time, speed up dissimilative oxidation. No polysaccharide accumulation during respiration of normal staphylococci has yet been detected, however.

Very few of the transformations incidental to growth are accomplished to any extent in these washed cell suspensions. Evidence has been found, however, of at least one reaction, blocked by gramicidin, that normally per-

forms work useful to the cell. Washed normal staphylococci, respiring in glucose, steadily remove inorganic phosphate from the medium, but cannot do so when small amounts of gramicidin are present (Table IX). In considering these experiments, it is important to keep certain points clearly in mind. The uptake does not refer to increase in phosphate esters, but to increase in total phosphorus sedimentable with the cells (as determined by the difference exhibited by the supernate). Also, the phosphate uptake is prevented by gramicidin although respiration is not. Of course, many poisons like mercuric chloride, halogen compounds, and most antiseptics inhibit phosphate uptake; but at the same time they prevent metabolic activity generally.

TABLE IX

EFFECT OF GRAMICIDIN ON OXYGEN AND PHOSPHATE UPTAKE
BY STAPHYLOCOCCI RESPIRING IN GLUCOSE

Molar concentration of phosphate	P present in medium, μg.	Without gramicidin		With gramicidin	
		O_2 uptake, μl.	P uptake, μg.	O_2 uptake, μl.	P uptake, μg.
0	0	107	0	151	0
0.0009	41	105	41	163	0.5
0.0022	95	105	42	160	0
0.0049	214	109	41	165	0
0.0097	420	111	39	172	0
0.0288	1250	113	ca. 43	227	0

There is some reason to believe that the effect of gramicidin upon phosphate uptake is more closely related to its bacteriostatic effect than is the effect upon oxygen uptake. It will be recalled that the bacteriostatic activity of gramicidin was low for certain resistant staphylococci and that it was diminished for ordinary strains by the presence of cephalin. These conditions have also an effect upon the metabolic action of gramicidin, reversing to a considerable extent its inhibition of phosphate uptake, but not so readily preventing its stimulatory action on oxygen uptake (Table X). On the other hand, absence of potassium and presence of ammonium ions are conditions that greatly affect the stimulation of respiration, but they have no material effect upon phosphate blocking by gramicidin. These results tend to make the stimulation of oxidation appear to be a secondary effect, which shows up only when the environmental conditions are favorable. All that can be said about the "phosphate-blocking effect" is that it occurs when bacteriostasis is possible and is diminished when

bacteriostasis is prevented.   That it should itself be the reason for growth inhibition is not made more likely by the observation that phosphate uptake by unpoisoned *washed* cells does not accompany respiration in all gramicidin-susceptible species.   On the other hand, it will be granted that

TABLE X

EFFECT OF GRAMICIDIN ON STAPHYLOCOCCI RESPIRING IN GLUCOSE:
CORRELATION OF BACTERIOSTATIC EFFECT WITH OXYGEN AND PHOSPHATE UPTAKE

Expt. No.	Modified condition	Resist- ance factor*	Without gramicidin		With gramicidin		Grami- cidin used, μg./ml.
			O_2, μl./hr.	P,** μg.	O_2, μl./hr.	P,** μg.	
1	Normal cells	1	70	36	152	4	25
	Phosphatidyl serine, 130 μg./ml.	30	67	31	141	19	25
	Ammonium chloride, 0.001 M	1	73	36	55	9	25
2	No potassium	1	81	25	85	4	25
	Potassium chloride, 0.012 M	1	85	32	169	0	25
3	Gramicidin-resistant						
	strain A	5	36	46	90	26	30
	strain B	20	26	18	26	12	20
	strain B	20	29	17	29	18	10

* Resistance factor = approximate bacteriostatic concentration of gramicidin divided by normal bacteriostatic concentration.

** Phosphate uptake is the total for a period of from 2–4 hours by 1–2 mg. of dry bacteria in the separate experiments.

all cells *when actually multiplying* must take up phosphorus by some means or other and that an agent which could prevent this process would presumably prevent growth.

MECHANISM OF PHOSPHATE UPTAKE.—That the phosphate uptake by staphylococci is a definite energy-using metabolic process is indicated by the following facts:

1.   It occurs only when substrate is being utilized.

2.   The phosphate uptake accompanying glucose oxidation is approximately proportional to the amount of oxygen taken up.

3.   Oxidation of glycerol, lactate, alcohol, or pyruvate is not accompanied by appreciable phosphate uptake.

4.   As shown in Table IX, the cells can remove the total phosphate present in the surrounding medium.

5.   The cells do not take up inorganic phosphate even in 0.33 molar phosphate buffer if substrate is not present.   Indeed, there have been a striking number of indications that phosphate uptake in a great variety of living cells is linked to metabolism.   Streptococci fermenting glucose (117), sulfur bacteria oxidizing sulfur (161), yeast growing in glucose (61, 83, 110), molds (96) and diatoms (80), and brain tissue (142) take up

phosphate in proportion to metabolic activity. Studies with phosphorus isotopes have indicated that exchange in muscle (46, 62, 138), erythrocytes (39, 131), and leukemic tissue (82) occurs chiefly under conditions favoring metabolic activity.

Concerning the mechanism of the phosphate-transferring reaction in staphylococci, the information is as yet scant. The phosphate that is taken up is about one-half recovered in inorganic form, a smaller part is labile (adenosinetri- or 7-minute) phosphate, and the remainder is in the form of various soluble esters. Gramicidin-treated cells do not increase their labile or adenosinetriphosphate phosphorus materially; but the other esters are somewhat increased, with a corresponding drop in the free inorganic phosphate (72). This is a good indication that the phosphorylating processes are still occurring, merely being obliged to operate with the quantity of phosphate already

TABLE XI

OXYGEN AND PHOSPHATE UPTAKE BY RESPIRING STAPHYLOCOCCI
IN PRESENCE AND ABSENCE OF IODOACETAMIDE

Iodoacetamide concn., M	Substrate	Total O_2 uptake, μl.	Total P uptake, μg.	$\dfrac{\mu M.\ O_2}{\mu M.\ P}$
0	Glucose	183	70	3.6
	Lactate	488	0	∞
	Glucose + lactate	503	137	5.1
0.003	Glucose	22	2	$ca.$ 15
	Lactate	313	0	∞
	Glucose + lactate	284	16	25
0 011	Glucose	22	0	∞
	Lactate	252	0	∞
	Glucose + lactate	214	14	21
0.013	None	20	0	∞
	Glycerol	16	0	∞
	Lactate	191	4	$ca.$ 70
	Glycerol + lactate	219	16	19

available inside the cell. Also, if the fraction determined as inorganic is true inorganic phosphate and not largely a labile ester—it does not have the properties of the acyl phosphates when fractionated by the method of Lipmann (89)—the phosphate does not seem to be s mply diffus ng into the cell in response to a low internal concentration resulting from esterification.

Some additional information is furnished by recent attempts toward further isolation of the phosphate-transferring reaction (72). As shown in Table XI, iodoacetamide prevents the oxidation of glucose and accompanying phosphate uptake. A definite but inefficient phosphate uptake will now occur, however, if lactate is being oxidized at the same time. It seems plausible to suppose that the pyruvate-to-acetate oxidation step makes possible phosphorylation of glucose (88), and establishes the phosphate-transferring system even though triose oxidation cannot occur.

Bearing in mind that processes such as "end oxidation" of glucose (25, 87, 164) may later prove to be the significant ones with these bacteria, we can yet say that the principal

known phosphate-utilizing reaction available to the iodoacetamide-poisoned cells is phosphorylase activity. Assuming a phosphorylase reaction analogous to that in animal tissues (24) and plants (47, 50):

$$\text{polysaccharide} + \text{inorganic phosphate} \rightleftarrows \text{glucose-1-phosphate}$$

we may suppose that a corresponding enzyme acting upon polysaccharide at or near the surface of normal cells can utilize external inorganic phosphate and thus introduce it, as a phosphorylated carbohydrate, inside the cell. Blocking of polysaccharide synthesis at this point or at one of the near-by steps by gramicidin would tend to inhibit (*a*) polysaccharide accumulation, (*b*) phosphate uptake, and (*c*) cell multiplication.

<div align="center">

TABLE XII

EFFECT OF GRAMICIDIN AND 2,4-DINITROPHENOL
ON PHOSPHATE UPTAKE BY BAKERS' YEAST RESPIRING IN GLUCOSE

</div>

Yeast suspension +	Phosphate uptake in μg. phosphorus at		
	30 min.	75 min.	180 min.
Control	440	715	925
Gramicidin, 90μg./ml.	475	705	890
Control	330	500	630
Dinitrophenol:			
M/22,000	170	320	370
M/5500	60	60	50
M/1100	−80	−80	−210

The above constitutes an hypothesis of the nature of gramicidin poisoning of staphylococci, but it cannot be completely evaluated at present. There are a few bits of supporting evidence to add. It will be recalled that Hoogerheide was originally working with tyrothricin because it inhibited production of capsular material (polysaccharide) by Friedländer's bacillus (67). Gramicidin has this effect in somewhat higher concentrations (40 μg./ml.), although growth of this Gram-negative bacillus is not affected (101). Indirect evidence has been presented that polysaccharide synthesis normally accompanying respiration is prevented by low concentrations of sodium azide or 2,4-dinitrophenol (21). These same poisons stimulate oxygen, and decrease phosphate, uptake by staphylococci. For example, respiration was 118, 137, 173, and 179%, and phosphate uptake 45, 11, 5, and 4% of normal in M/650 azide, M/130 azide, M/4000 dinitrophenol, and M/650 dinitrophenol, respectively. An experiment carried out with yeast (Table XII) is even of more interest because, in this species, polysaccharide synthesis and blocking of synthesis have been demonstrated by actual analyses (119, 120). It will be seen that dinitrophenol has a marked inhibitory effect on phosphate uptake by yeast, while gramicidin has not. Whether or not this is related to the fact that yeast growth is not much affected by gramicidin, it is clear that species differences, be they differences in permeability, inhibitory substances, structures synthesized, or enzymes performing the synthesis, will play a large part in determining susceptibility. It must be added here that dinitrophenol, while definitely bacteriostatic for staphylococci at

$M/5000$, even in much higher concentrations allowed survival of resistant organisms, which eventually grew out heavily. Gramicidin, provided in excess, is more definitely bactericidal than this.

The observation of the effects of dinitrophenol on phosphate metabolism may be a contribution to the understanding of that agent. Little that applies to the present problem is known about the intimate mechanism of its action. Vandendriessche has reported that it activates the formation of hexosediphosphate from the monophosphate in yeast juice (160), a result not inconsistent with the experiments in muscle (23). This appears to provide one mechanism for the shift in the direction away from polysaccharide synthesis. One potentiality gramicidin and dinitrophenol may be supposed to have in common, that of being hydrolyzed or reduced to a 1,2-aminohydroxy compound, does not explain their apparent similarity of action, since the appropriate aminophenols do not have any activity toward staphylococci (72).

It has been suggested that analogous phosphate-uptake processes may be common to many types of cells. Since the effects of gramicidin on respiration of bacteria as described above have parallels in its effects on bovine spermatozoa (53), on muscle (71), and apparently also on frog embryos and erythrocytes (71), it may well be that gramicidin is potentially capable of affecting most cells. The existence of inhibitors such as the cephalins would conceivably explain why it, in contrast to the somewhat similarly acting dinitrophenol, is selective in its action to a much greater degree.

4. Mode of Action of Tyrocidine

Tyrocidine is bactericidal in its action, bacteriolysis occurring with some cells, such as pneumococci and staphylococci and some Gram-positive bacilli (34). Dubos has suggested that the lysis is secondary to a more direct injury that releases the activity of an autolytic system if such is present (29). The nature of the primary injury by tyrocidine or other "lytic" agents has not as yet been ascertained.

It has recently been found, as Table XIII reveals, that soluble cell constituents are released from bacteria by tyrocidine (73). Extraction of cells for 5 minutes at 37° C. with 0.017% tyrocidine hydrochloride was about as effective as prolonged extraction of the cells with 5% trichloroacetic acid at 0° C. Longer exposure to tyrocidine resulted in release of more nitrogen and phosphorus compounds, undoubtedly as the result of autolytic processes. The streptococci, as would be predicted, did not exhibit any cellular lysis; the staphylococci exhibited a slow decrease in optical density of 3% per hour at 0° C. and 18% per hour at 37° C.

These experiments clearly show that bacteriolysis by tyrocidine is a secondary phenomenon, following slowly after a rapid primary injury which is apparently some alteration of the cellular membrane or surface. As a result, soluble compounds can be leached out of even a resistant cell like the streptococcus, at low temperatures. It is likely that this is adequate to explain cessation of metabolic activity, since substrates and co-

enzymes are presumably enormously diluted in the process. An inhibition is also in some way released so that, to the extent possible, autolysis of the cell proceeds in a fashion characteristic of the cell.

It is of interest that neither the bacteriostatic basic protein of wheat (5), which was reported not to have lytic activity (153), nor gramicidin, has the same effect as tyrocidine on the bacteria. In other experiments, it was

TABLE XIII

NITROGEN AND PHOSPHORUS RELEASED FROM BACTERIA BY TYROCIDINE
AND OTHER AGENTS

Agent	Concn., μg./ml.	Incubation		Analysis of supernate			
		Temp., °C.	Time, min.	Total N, mg.	Inorg. P, μg.	Labile P, μg.	Ester P, μg.
Staphylococcus aureus							
Control	...	0	20	0.14	21	0	0
	...	37	30	0.27	33	0	0
Tyrocidine	165	0	10	0.64	207	4	33
	165	37	5	1.07	283	4	25
	165	37	30	1.32	313	18	99
	165	37	90	2.41	367	7	443
Gramicidin	165	37	30	0.20	53	0	0
Wheat protein (5)	330	37	30	0.11	47	7	0
Trichloroacetic acid, 5%	...	0	90	1.10	280	..	48
Streptococcus hemolyticus (Group D)							
Control	...	37	30	0.68	50	0	6
Tyrocidine	165	0	10	0.72	137	4	39
	165	37	10	0.88	200	8	63
	165	37	30	1.44	232	5	61
Gramicidin	165	37	30	0.31	50	0	7
Trichloroacetic acid, 5%	...	0	30	..	127	0	...

found that acetylation of tyrocidine virtually eliminated both the bactericidal activity and the effect on cell surfaces. Furthermore, various cationic and anionic surface-active agents, including Duponol C, hexylresorcinol, fixanol, cetylmalonic acid, and Sapamine KW, have given the same effect as tyrocidine, in about the same amounts (73). We may conclude that tyrocidine, by virtue of its basic groups, is a bactericidal detergent, unique only in its natural origin and complexity of chemical composition.

5. The Bacterial Cell as a Part of the Mechanism

The experiments described illustrate that the search for an explanation of the mechanisms by which antibacterial agents produce their effects is likely to lead one into a study of normal bacteria. Those properties which seem characteristic of any living cell are:

1.   The ability to transform various substrates found in a favorable environment into characteristic end products and to liberate chemical energy in so doing.

2.   The ability to use this chemical energy for synthesizing other substrate molecules into the typical constituents of living protoplasm.

3.   Maintenance of the organized transforming and synthesizing systems inside a cell membrane or retaining surface.

4.   The ability to transport various needed substrates and metabolic products selectively across the boundaries of the system.

As abstract functions these are distinct enough, but as biochemical and biophysical mechanisms they undoubtedly greatly overlap.

Most of what we know about bacterial physiology is related to property (1) above rather than the other energy-using, functions. It is natural, therefore, that each new antibacterial agent is first tested for effect upon these degradative metabolic functions. Typical antiseptics like the mercury, copper, iodine, chlorine derivatives, the phenols, and detergents, have been found to interfere with the degradative processes. Evidence has been presented above suggesting that gramicidin affects rather some energy-using process (2 or 4 above) which would normally allow carbohydrate and phosphate storage. It is beginning to appear likely that effective chemotherapeutic agents like the sulfonamides and penicillin probably also affect the way in which the susceptible cell uses the energy rather than the processes by which energy is liberated. We can say that sulfanilamide, sulfapyridine, sulfathiazole, penicillin, and the bacteriostatic wheat protein (5) do not in any case have the same effects as gramicidin upon the phosphate metabolism of staphylococci (72).

Evidence has also been presented to indicate that tyrocidine, and a number of detergents, so modify the cell surface (function 3) that vital soluble metabolites such as nitrogen compounds, inorganic phosphate, and phosphate esters are washed out of the cell.

Although the basic phenomena of antibacterial action have to be observed in a nutrient medium where growth is possible, in such media the metabolism of poisoned cells, which do not grow, deviates from that of normal cells by a host of qualitative and quantitative differences, most of

which are results, and not to be construed as causes, of growth inhibition. Washed bacteria in buffer suspensions must perforce occupy themselves either with rearrangement of their own cellular material or utilization of whatever substrates the experimenter provides. If one is able under these circumstances to detect an alteration of metabolism, there is much more chance that it is, if not the primary injury, at least in the chain of causal events antecedent to the effect on cell growth. To determine more conclusively that the observed injury is actually a cause, it will be necessary to work back toward more adequate conditions for growth. To determine what injury is the primary one, it will be necessary to work toward simpler systems and the use of metabolizing cell extracts and enzyme preparations.

It is here that the bacterial physiologist should contribute much toward our knowledge of the manner in which antibacterial agents act. In return, it is likely that the "antibacteriologist" will be able to provide the physiologist with an ever-increasing number of selective agents which in low concentration exert well-defined specific effects upon protoplasm.

Bibliography

1. Allcroft, R., *Vet. Bull.*, **12**, 1 (1942).
2. Ark, P. A., and Hunt, M. L., *Science*, **93**, 354 (1941).
3. Auerswald, H., *Zentr. Bakt. Parasitenk., I, Orig.*, **142**, 32 (1938).
4. Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exptl. Med.*, **74**, 621 (1941).
5. Balls, A. K., Hale, W. S., and Harris, T. H., *Cereal Chem.*, **19**, 279 (1942).
6. Bean, C. W., Miller, W. T., and Heishman, J. O., *Vet. Med.*, **37**, 401 (1942).
7. Bellows, J. G., *Trans. Am. Acad. Ophthalmol.*, **47**, 19 (1942).
8. Bergmann, M., and Stein, W. H., *J. Biol. Chem.*, **129**, 609 (1939).
9. Besta, B., and Kuhn, H., *Z. Hyg. Infektionskrankh.*, **116**, 520 (1934).
10. Blair, J. E., and Hallman, F. A., *J. Infectious Diseases*, **72**, 246 (1943).
11. Boivin, A., *Ann. inst. Pasteur*, **61**, 426 (1938).
12. Bordley, J. E., Crowe, S. J., Dolowitz, D. A., and Pickrell, K. L., *Ann. Otol. Rhin. Laryngol.*, **51**, 936 (1942).
13. Brewer, C. M., *Am. J. Pub. Health*, **33**, 261 (1943).
14. Bryan, C. S., Horwood, R. E., and Clark, C. F., *J. Dairy Sci.*, **25**, 713 (1942).
15. Bryan, C. S., Weldy, M. L., and Greenberg, J., *Vet. Med.*, **37**, 364 (1942).
16. Canneyt, J. van, *Compt. rend. soc. biol.*, **95**, 878 (1926).
17. Chambers, S. O., and Weidman, F. D., *Arch. Dermatol. Syphilol.*, **18**, 568 (1928).
18. Christensen, H. N., *J. Biol. Chem.*, **151**, 319 (1943).
19. Christensen, H. N., Edwards, R. R., and Piersma, H. D., *ibid.*, **141**, 187 (1941).
20. Clapp, M. P., *Proc. Soc. Exptl. Biol. Med.*, **51**, 279 (1942).
21. Clifton, C. E., and Logan, W. A., *J. Bact.*, **37**, 523 (1939).
22. Cordon, T. C., and Haenseler, C. M., *Soil Sci.*, **47**, 207 (1939).
23. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **116**, 119 (1936).
24. Cori, G. T., and Cori, C. F., *ibid.*, **135**, 733 (1940).
25. Dickens, F., *Biochem. J.*, **32**, 1626 (1938).

26. Dimick, K. P., *J. Biol. Chem.*, **149**, 387 (1943).
27. Doherty, D. G., Stein, W. H., and Bergmann, M., *ibid.*, **135**, 487 (1940).
28. Downs, C. M., *J. Bact.*, **45**, 137 (1943); **44**, 392 (1942).
29. Dubos, R. J., *J. Exptl. Med.*, **70**, 1 (1939).
30. Dubos, R. J., *ibid.*, **70**, 11 (1939).
31. Dubos, R. J., *Ann. Internal Med.*, **13**, 2025 (1940).
32. Dubos, R. J., and Cattaneo, C., *J. Exptl. Med.*, **70**, 249 (1939).
33. Dubos, R. J., Coburn, A. F., and Hotchkiss, R. D., *unpublished results*, 1941.
34. Dubos, R. J., and Hotchkiss, R. D., *J. Exptl. Med.*, **73**, 629 (1941).
35. Dubos, R. J., and Hotchkiss, R. D., *Trans. Coll. Physicians Phila.*, (4) **10**, 11 (1942).
36. Dubos, R. J., Hotchkiss, R. D., and Coburn, A. F., *J. Biol. Chem.*, **146**, 421 (1942).
37. Edwards, S. J., *Proc. Roy. Soc. Med.*, **35**, 632 (1942).
38. Eichhorn, A., *J. Am. Vet. Med. Assoc.*, **100**, 7 (1942).
39. Eisenman, A. J., Ott, L., Smith, P. K., and Winkler, A. W., *J. Biol. Chem.*, **135**, 165 (1940).
40. Folch, J., *ibid.*, **146**, 35 (1942).
41. Fosdick, L. S., and Rapp, G. W., *Arch. Biochem.*, **1**, 379 (1943).
42. Francis, A. E., *Lancet*, **1942 I**, 408.
43. Franke, H., and Ismet, A., *Centr. Bakt. Parasitenk., I, Orig.*, **99**, 570 (1926).
44. de Freudenreich, E., *Ann. inst. Pasteur*, **2**, 200 (1888).
45. Friedemann, T. E., *J. Biol. Chem.*, **130**, 61 (1939).
46. Furchgott, R. F., and Shorr, E., *Federation Proc.*, **1**, 27 (1942).
47. Green, D. E., and Stumpf, P. K., *J. Biol. Chem.*, **142**, 355 (1942).
48. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 86 (1943).
49. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *ibid.*, **37**, 313 (1943).
50. Hanes, C. S., *Proc. Roy. Soc.*, London, **B129**, 174 (1940).
51. Heilman, D., and Herrell, W. E., *Proc. Soc. Exptl. Biol. Med.*, **46**, 182 (1941).
52. Heilman, D., and Herrell, W. E., *ibid.*, **47**, 480 (1941).
53. Henle, G., and Zittle, C. A., *ibid.*, **47**, 193 (1941).
54. Herrell, W. E., *Trained Nurse and Hospital Rev.*, **110**, 114 (1943).
55. Herrell, W. E., and Heilman, D., *J. Clin. Investigation*, **20**, 583 (1941).
56. Herrell, W. E., and Heilman, D., *J. Am. Med. Assoc.*, **118**, 1401 (1942).
57. Herrell, W. E., and Heilman, D., *Am. J. Med. Sci.*, **206**, 221 (1943).
58. Herrell, W. E., Heilman, D., and Gage, R. P., *ibid.*, **206**, 26 (1943).
59. Herrell, W. E., and Heilman, D., *ibid.*, **205**, 157 (1943).
60. Hettche, H. O., and Weber, B., *Arch. Hyg. Bakt.*, **123**, 69 (1940).
61. Hevesy, G., Linderstrøm-Lang, K., and Nielsen, N., *Nature*, **140**, 725 (1937).
62. Hevesy, G., and Rebbe, O., *Acta Physiol. Scand.*, **1**, 171 (1940).
63. Hobby, G. L., Meyer, K., and Chaffee, E., *Proc. Soc. Exptl. Biol. Med.*, **50**, 281 (1942).
64. Holiday, E. R., *Biochem. J.*, **30**, 1795 (1936).
65. Holiday, E. R., and Ogston, A. G., *ibid.*, **32**, 1166 (1938).
66. Holiday, E. R., *private communication*, 1943.
67. Hoogerheide, J. C., *J. Bact.*, **40**, 415 (1940).
68. Hoogerheide, J. C., *J. Franklin Inst.*, **229**, 677 (1940).
69. Horwood, R. E., Clark, C. F., and Bryan, C. S., *J. Dairy Sci.*, **25**, 714 (1942).

70. Hotchkiss, R. D., *J. Biol. Chem.*, **141**, 171 (1941).
71. Hotchkiss, R. D., *J. Bact.*, **45**, 64 (1944); and *unpublished results*.
72. Hotchkiss, R. D., to be published, 1946.
73. Hotchkiss, R. D., in preparation, 1946.
74. Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, **132**, 791 (1940).
75. Hotchkiss, R. D., and Dubos, R. J., *ibid.*, **132**, 793 (1940).
76. Hotchkiss, R. D., and Dubos, R. J., *ibid.*, **136**, 803 (1940).
77. Hotchkiss, R. D., and Dubos, R. J., *ibid.*, **141**, 155 (1941).
78. Katznelson, H., *J. Bact.*, **39**, 101 (1940).
79. Katznelson, H., *Can. J. Research*, **C20**, 169 (1942).
80. Ketchum, B. H., *J. Cellular Comp. Physiol.*, **13**, 373 (1939).
81. Kimmelstiel, P., *Centr. Bakt. Parasitenk.*, *I, Orig.*, **89**, 113 (1923).
82. Lawrence, J. H., Tuttle, L. W., Scott, K. G., and Connor, C. L., *J. Clin. Investigation*, **19**, 267 (1940).
83. Lawrence, J. H., Erf, L. A., and Tuttle, L. W., *J. Applied Phys.*, **12**, 333 (1941).
84. LePage, G. A., *Arch. Biochem.*, **1**, 255 (1942).
85. LePage, G. A., and Umbreit, W. W., *J. Biol. Chem.*, **148**, 255 (1943).
86. Levaditi, C., and Twort, C., *Compt. rend. soc. biol.*, **70**, 645, 753, 799 (1911).
87. Lipmann, F., *Nature*, **138**, 588 (1936).
88. Lipmann, F., *ibid.*, **143**, 281 (1939).
89. Lipmann, F., *J. Biol. Chem.*, **134**, 463 (1940).
90. Lipmann, F., Hotchkiss, R. D., and Dubos, R. J., *ibid.*, **141**, 163 (1941).
91. Little, R. B., *16th Ann. Rept. N. Y. State Assoc. Milk Sanitarians*, 107 (1942).
92. Little, R. B., Dubos, R. J., and Hotchkiss, R. D., *J. Am. Vet. Med. Assoc.*, **98**, 189 (1941); *Proc. Soc. Exptl. Biol. Med.*, **44**, 444 (1940); **45**, 462 (1940).
93. Little, R. B., Dubos, R. J., Hotchkiss, R. D., Bean, C. W., and Miller, W. T., *Am. J. Vet. Research*, **2**, 305 (1941).
94. Lohmann, K., *Biochem. Z.*, **203**, 164 (1928).
95. MacLeod, C. M., Mirick, G. S., and Curnen, E. C., *Proc. Soc. Exptl. Biol. Med.*, **43**, 461 (1940).
96. Mann, T., *Nature*, **151**, 619 (1943).
97. Mann, F. C., Heilman, D., and Herrell, W. E., *Proc. Soc. Exptl. Biol. Med.*, **52**, 31 (1943).
98. Marie, A.-C., *Compt. rend. soc. biol.*, **92**, 561 (1925).
99. Martin, F. E., *J. Am. Vet. Med. Assoc.*, **101**, 23 (1942); **102**, 267 (1943).
100. Martin, F. E., *Vet. Med.*, **38**, 174 (1943).
101. McDonald, E., *J. Franklin Inst.*, **229**, 805 (1940).
102. McIntosh, J., and Selbie, F. R., *Lancet*, **1942 II**, 750.
103. Miller, B. F., Abrams, R., Dorfman, A., and Klein, M., *Science*, **96**, 428 (1942).
104. Moore, S., Stein, W. H., and Hotchkiss, R. D., *unpublished results*, 1942.
105. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **150**, 113 (1943).
106. Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, **34**, 169 (1940).
107. Much, H., *Münch. med. Wochschr.*, **72**, 374 (1925).
108. Much, H., and Sartorius, F., *Med. Klin.*, **20**, 347 (1924).
109. Mulchinock, W. J., *Can. J. Comp. Med.*, **6**, 95 (1942).
110. Mullins, L. J., *Biol. Bull.*, **83**, 326 (1942).
111. Neter, E., *Proc. Soc. Exptl. Biol. Med.*, **49**, 163 (1942); **51**, 256 (1942).
112. Neter, E., *Science*, **96**, 209 (1942).

113. Nicolle, M., *Ann. inst. Pasteur*, **21**, 613 (1907).
114. Nielsen, N., and Johansen, G., *Naturwissenschaften*, **31**, 235 (1943).
115. Ogston, A. G., *Trans. Faraday Soc.*, **39**, 151 (1943).
116. Ogston, A. G., *private communication*, 1943.
117. O'Kane, D. J., and Umbreit, W. W., *J. Biol. Chem.*, **142**, 25 (1942).
118. Phillips, R. L., and Barnes, L. H., *J. Franklin Inst.*, **233**, 396 (1942).
119. Pickett, M. J., and Clifton, C. E., *Proc. Soc. Exptl. Biol. Med.*, **46**, 443 (1941).
120. Pickett, M. J., and Clifton, C. E., *J. Cellular Comp. Physiol.*, **21**, 77 (1943).
121. Pico, C. E., *Rev. inst. bacteriol. "Carlos G. Malbran,"* **10**, 166 (1941).
122. Pomerat, C. M., *Proc. Soc. Exptl. Biol. Med.*, **51**, 345 (1942).
123. Pringsheim, E. G., *Centr. Bakt. Parasitenk.*, *II*, **51**, 72 (1920).
124. Pulvertaft, R. J. V., *Lancet*, **1943 II**, 1.
125. Rake, G., Jones, H., and McKee, C. M., *Proc. Soc. Exptl. Biol. Med.*, **52**, 136 (1943).
126. Rammelkamp, C. H., *ibid.*, **49**, 346 (1942).
127. Rammelkamp, C. H., *War Med.*, **2**, 830 (1942).
128. Rammelkamp, C. H., *J. Infectious Diseases*, **71**, 40 (1942).
129. Rammelkamp, C. H., and Weinstein, L., *Proc. Soc. Exptl. Biol. Med.*, **48**, 211 (1941).
130. Rammelkamp, C. H., and Weinstein, L., *J. Infectious Diseases*, **71**, 166 (1942).
131. Rapoport, S., Leva, E., and Guest, G. M., *J. Biol. Chem.*, **139**, 633 (1941).
132. Robinson, H. J., and Graessle, O. E., *J. Pharmacol.*, **76**, 316 (1942).
133. Robinson, H. J., and Molitor, H., *ibid.*, **74**, 75 (1942).
134. Robson, J. M., and Scott, G. I., *Lancet*, **1943 I**, 100.
135. Rodaniche, E. C., and Palmer, W. L., *J. Infectious Diseases*, **72**, 154 (1943).
136. Rosenthal, L., *Compt. rend. soc. biol.*, **92**, 78, 472 (1925); **93**, 1569 (1925).
137. Rosenthal, L., *Proc. Soc. Exptl. Biol. Med.*, **46**, 448 (1941).
138. Sacks, J., and Altshuler, C. H., *Am. J. Physiol.*, **137**, 750 (1942).
139. Sanfelice, F., *Arch. Hyg. Bakt.*, **110**, 348 (1933).
140. Sartorius, F., *Centr. Bakt. Parasitenk.*, **93**, 162 (1924).
141. Scales, F. M., and Kemp, M., *Intern. Assoc. Milk Dealers, Assoc. Bull.*, **33**, 491 (1941).
142. Schachner, H., Fries, B. A., and Chaikoff, I. L., *J. Biol. Chem.*, **146**, 95 (1942).
143. Schalm, O. W., *J. Am. Vet. Med. Assoc.*, **100**, 323 (1942).
144. Schoenbach, E. B., Enders, J. F., and Mueller, J. H., *Science*, **94**, 217 (1941).
145. Schoenbach, E. B., and Seidman, L. R., *Proc. Soc. Exptl. Biol. Med.*, **49**, 108 (1942).
146. Sirotinin, *Z. Hyg. Infektionskrankh.*, **4**, 262 (1888).
147. Stein, W. H., Moore, S., Stamm, G., Chou, C-Y., and Bergmann, M., *J. Biol. Chem.*, **143**, 121 (1942).
148. Stokes, J. L., Peck, R. L., and Woodward, C. R., *Proc. Soc. Exptl. Biol. Med.*, **51**, 126 (1942).
149. Stokes, J. L., and Woodward, C. R., *J. Bact.*, **43**, 253 (1942).
150. Stokes, J. L., and Woodward, C. R., *ibid.*, **46**, 83 (1943).
151. Stokinger, H. E., Ackerman, H., and Carpenter, C. M., *ibid.*, **45**, 31 (1943).
152. Stone, R. W., and Werkman, C. H., *Biochem. J.*, **31**, 1516 (1937).
153. Stuart, L. S., and Harris, T. H., *Cereal Chem.*, **19**, 288 (1942).

154. Synge, R. L. M., Gordon, A. H., and Martin, A. J. P., *private communication,* 1943.

155. Tillett, W. S., Cambier, M. J., and Harris, W. J., *J. Clin. Investigation,* **22,** 249 (1943).

156. Tishler, M., Stokes, J. L., Trenner, N. R., and Conn, J. B., *J. Biol. Chem.,* **141,** 197 (1941).

157. Tripp, L. H., and Lawrence, C. A., *Cornell Vet.,* **32,** 90 (1942).

158. Udall, D. H., Johnson, S. D., and Ferguson, J., *ibid.,* **33,** 209 (1943).

159. Valette, G., Cavier, R., and Liber, A., *Compt. rend. soc. biol.,* **135,** 849 (1941).

160. Vandendriessche, L., *Enzymologia,* **10,** 69 (1941).

161. Vogler, K. G., and Umbreit, W. W., *J. Gen. Physiol.,* **26,** 157 (1942).

162. Waksman, S. A., *Bact. Revs.,* **5,** 231 (1941).

163. Waksman, S. A., and Woodruff, H. B., *J. Bact.,* **44,** 373 (1942).

164. Warburg, O., and Christian, W., *Biochem. Z.,* **292,** 287 (1937).

165. Weiland, P., *Zentr. Bakt. Parasitenk., I, Orig.,* **136,** 451 (1936); **147,** 321 (1941).

166. Weinstein, L., and Rammelkamp, C. H., *Proc. Soc. Exptl. Biol. Med.,* **48,** 147 (1941).

167. Willstätter, R., and Rohdewald, M., *Z. physiol. Chem.,* **247,** 269 (1937).

168. Wolf, J. E., *Zentr. Bakt. Parasitenk., I, Orig.,* **84,** 241 (1920).

169. Woolley, D. W., and Krampitz, L. O., *J. Biol. Chem.,* **146,** 273 (1942).

170. Wright, V. W. M., *J. Franklin Inst.,* **233,** 188 (1942).

171. Zukerman, I., and Minkewitsch, I., *Zentr. Bakt. Parasitenk., I, Ref.,* **80,** 483 (1925).

.

BIOLOGICAL ENERGY TRANSFORMATIONS AND THE CANCER PROBLEM

By

V. R. POTTER

Madison, Wisconsin

CONTENTS

I. Introduction

Current trends in cancer research indicate a growing realization that cancer tissues are abnormal in respect to one or more enzymes which go to make up the total mosaic of interlocking biocatalysts responsible for the metabolism of living tissues. The application of organized enzyme re-

search to the cancer problem has of course been delayed by the lack of agreement as to the significance of enzyme studies on normal tissues; but within the past two or three years a relatively large area of agreement has been developing in the field of energy transformations, which involve the phosphorylative, glycolytic, and respiratory enzyme systems. The present review will be limited to a discussion of these three enzyme systems, which are so closely integrated that it would not be possible to discuss any one of the three without mentioning the others. This limitation in scope is especially desirable since Greenstein (86*) reviewed the broader aspects of tumor enzymology in 1943, and since the existing knowledge regarding the action of these three groups of enzymes in normal tissues can be organized into a definite pattern. It is only by such organization that the significance of any specific enzymatic defect in cancer tissue can be appreciated.

By way of orientation it seems appropriate to quote the report (16*) of a committee of leading authorities who, in 1938, drew the following conclusions regarding the nature of cancer:

"1. Malignancy is a universal cell potentiality, in that any cell has inherent in its make-up the potentiality for unlimited or uncontrolled growth.

"2. The degree of this potentiality for malignancy is a variable quantity for each tissue or cell type, and this degree is determined largely, if not entirely, by heredity or predisposing factors.

"3. The malignancy potentiality of a cell may be developed in the more sensitive groups by the strain of normal physiologic processes but may be set off even in resistant groups by a variety of inciting agents.

"4. The change from a normal to a malignant cell represents an alteration in the cell itself by virtue of which proliferation becomes an automatic process independent of a continuously acting provocative agent.

"5. The new property of the cell appears to develop suddenly, becomes a fixed character, and is transmitted to its descendants. It gives evidence of being a somatic mutation."

These five postulates represent the more or less solid ground of positive achievement in the cancer field, and a considerable advance in the study of this problem. The negative side is illustrated by the additional comments of Murphy (146*):

"It is probable that substantial advancement in the methods of treatment will come only with a fuller knowledge of the formal genesis or the mechanism by which the growth capacity of the cancer is maintained. Unfortunately, this important part of the cancer problem is still little understood. If some substantial specific characteristics of the cancer cell could be established, it would serve as an opening lead; but no such character, other than the capacity for growth, has yet been found. . . .At the present time there is no theory as to the nature of cancer sufficiently comprehensive to be taken seriously."

With reference to the uncontrolled growth of cancer cells, the committee (16*) raised the question "Have they become 'fast' to the conditions which normally *control* cell growth in the body or is there a break in the internal *control mechanism* of the cell, or is

there a loss in body *control* of cell activity? This, the core of the problem, has been almost entirely neglected." [Reviewer's italics.] In response to this challenge, Berrill in a 1943 review entitled *Malignancy in Relation to Organization and Differentiation* (23*) stated:

"The apparent neglect is clearly not lack of consideration. It is due to the standards of reference also being problems as challenging as malignancy itself. Malignancy and its associated phenomena merely constitute one aspect of growth, proliferation and differentiation of cells and cell constituents. The nature of the growth controlling forces of the body, of the internal control mechanism of the cell, the relation between nuclear change and cytoplasmic and organismic expression, the determination and maintenance of cellular specializations, and the mode of reproduction of proteins, mitochondria, enzymes, genes, and viruses are as little understood and equally challenging. The problem of malignancy becomes one of synthesis involving the problems of development as a whole."

The literature cited (23*) presents the enzymologist with a wealth of material, which constitutes the biological mechanisms and correlations that have been worked out; but it is evident that enzyme chemistry has not been extensively applied to these problems, and Berrill concludes that "the problem seems to be infinitely complex and appears to be the nature of protein synthesis and reproduction, its relationships to cell surface qualities and general metabolism, and the susceptibility of the whole to *the inhibitory control of organization,* whatever the basis of this may be." [Reviewer's italics.]

The fact that enzyme chemistry has not solved these problems is not because enzyme studies have no contribution to make, but because enzyme techniques have not been sufficiently applied. Enzyme chemists have quite rightly taken the view that the fundamentals need to be understood before the various applications can be made. However, there is enough information at hand even now to permit a certain amount of organization, and one is justified in attempting to place this material in relation to the problems (16*, 23*, 146*) which confront the cancer investigator. The reviewer has constantly had in mind the need for the elucidation of *control mechanisms,* and the conviction that these mechanisms must be enzymatic in nature. In attempting to bring various facts into perspective, rather elaborate charts have had to be worked out. It is hoped that these will assist in showing the interrelationships of the various enzyme systems. In the preparation of this manuscript, extensive use has been made of numerous reviews which have appeared both in *Advances in Enzymology* and elsewhere, and the bibliography has been greatly shortened by reference in many instances to such reviews. Throughout the text these reviews or general references will be marked with an asterisk. There are undoubtedly many pieces of original literature which have been unintentionally overlooked.

The review will not deal with metabolic studies of the Warburg type, since these have been adequately discussed by Burk (32*, 33*, 34) and by Elliott (65*, 68) and subsequent publications have not altered the situation. There is general agreement that both anaerobic glycolysis ($Q_{CO_2}^{N_2}$) and aerobic glycolysis ($Q_{CO_2}^{O_2}$) are high in cancer tissue, but Warburg's original finding of a low Q_{O_2} has been modified by the finding that numerous cancers have essentially normal Q_{O_2} values, under the conditions

usually employed in the Warburg studies, that is—using the slice technique and with the Q_{O_2} based on no substrate or with glucose added. Regarding the question of whether the R.Q. is low or normal in tumor tissue, the reader is referred to the discussions cited (32*, 33*, 34, 65*, 68). We have previously pointed out (61) the limitations of the older approach, which was useful in orienting later enzyme studies, but which can now be supplemented by more specific measurements. That the over-all Q_{O_2} of a pathological tissue can be identical with its normal counterpart was well demonstrated by the study of nephritic kidney respiration made by Lyman and Barrón (130). These workers found that the average endogenous Q_{O_2} values were 17.6 ± 0.4 for both the normal and the nephritic kidneys. On the other hand, the oxidation of pyruvate was reduced from 8.2 to 0.5, while the lactate Q_{O_2} was reduced from 7.7 to 1.0 in the normal and in the nephritic kidneys, respectively. These data show that fundamental changes in the enzymatic constitution of a tissue can take place without affecting the resting Q_{O_2}. Similar results have been obtained in the case of tumor tissues.

Elliott and coworkers have published a series of papers (19, 64, 67, 69, 70, 71, 72, 74, 87) which have involved the use of various substrates in addition to glucose; and they have also carried out analyses to determine the extent of substrate disappearance. Such analytical studies are of course essential in this type of investigation, as Krebs' work has emphasized (115*). When the Q_{O_2} value of a tissue is raised to a new value by the addition of substrate, it is not legitimate to assume that the ΔQ_{O_2} is due to the oxidation of the added substrate, or even to the oxidation of metabolites derived from it, because, unless a comprehensive balance sheet is set up, one cannot know to what extent the endogenous Q_{O_2} has been supplanted by the oxidation of the added substrate. The studies using sliced or minced tissue with various substrates imply that, if the Q_{O_2} on a particular substrate is low, the enzyme system responsible for the oxidation of the substrate is low; but this can only be an assumption until the specific enzyme is studied. Nevertheless, the papers of Elliott *et al.*, serve as extremely valuable guides for the orientation of more specific enzyme studies. His demonstration (69) of a lowered succinic acid Q_{O_2} in two types of tumor which respired at a rate comparable with normal tissue has been confirmed and extended by Salter and coworkers (53), as well as by Elliott's own studies using homogenized tissue (71) and by Schneider and Potter (189), who have developed an assay method specific for succinic dehydrogenase (190). The observation of Elliott and Greig (70) that pyruvate is not converted to succinate in the two tumors which were studied merits

further investigation now that the path of this conversion is somewhat better understood.

The studies of Salter, *et al.* (53) on the succinate system deserved closer·discussion in this review both because of their emphasis on the deficiency of succinate-oxidizing enzymes in tumors and because of their demonstration of the limitations of Q_{O_2} determinations alone. These workers were extremely careful to choose tumors for which homologous control tissues were available. Some of their data have been summarized in Table I, in which it is seen that, for muscle and liver tumors, the no-substrate Q_{O_2} values of the tumors are not lowered but that, in the presence of added succinate, the normal tissues have a much greater Q_{O_2} than the tumor tissues. These data show that the usual Q_{O_2} values alone would not have revealed a deficiency in the tumor tissue in these instances.

TABLE I

OXYGEN CONSUMPTION OF NORMAL AND MALIGNANT HOMOLOGOUS TISSUE SLICES
WITH AND WITHOUT SUCCINATE SUBSTRATE (53)

Normal tissue			Malignant tissue		
Type	Endogenous Q_{O_2}	Succinate Q_{O_2}	Type	Endogenous Q_{O_2}	Succinate Q_{O_2}
Mouse liver	10.6	25.2	Mouse hepatoma	10.5	12.2
Mouse muscle (diaphragm)	7.0	20.9	Rhabdomyosarcoma	8.7	12.2

Our own experience with the oxidation of succinate in mouse-liver homogenates suggests that their Q_{O_2} values on sliced tissue have limited possibilities of interpretation even when succinate is added, since we have obtained succinate Q_{O_2} values (unpublished) in homogenized mouse liver in the range of 120 to 150 as compared to the value of 25.2 reported in Table I. In the case of rat liver, Rosenthal (178) obtained succinate Q_{O_2} values of 90–100 provided the slices were about 0.12 mm. in thickness and the succinate concentration was 0.04 *M*. Under these conditions, the rate of oxygen uptake is probably determined by the succinic dehydrogenase concentration, because the Q_{O_2} values approximate those obtained with the succinic dehydrogenase test system (189, 190). These results suggest that the succinate Q_{O_2} in the normal tissues studied by Craig, Bassett, and Salter (53) was not limited by the succinic dehydrogenase concentration, as might be inferred, but rather by the substrate concentration or by the rate of oxygen diffusion, and possibly by the cytochrome c concentration.

While much information can be obtained by means of the slice technique,

and in fact must be obtained by this technique in cases where the metabolic pathways or enzymatic components are unknown, it is obvious that where the pathways and components *are* known, it is desirable to investigate tumors in terms of these specific components. It is also apparent that, where these questions are still in dispute, the attempts to study tissues in terms of specific enzymatic components will do much to clarify the issue. Thus, the Krebs cycle (see below) can be demonstrated in all its complexity in only a few tissues. The demonstration of each of the component parts of the cycle would do much to settle the question as to what extent this mechanism applies in other tissues.

II. Normal Mechanisms of Energy Transfer

1. Glycolytic Mechanisms: Metabolic Pathways

Glycolysis is the process by which carbohydrate, including glycogen, glucose, fructose, or mannose, is broken down to lactic or pyruvic acid. The mechanism of glycolysis is the framework with which subsequent knowledge of oxidative and phosphorylative mechanisms must be integrated. It is therefore desirable to establish the nature of the normal glycolytic process and determine whether or not tumors utilize this mechanism before we discuss the reactions which stem from this basic metabolic pathway.

The well-established fact that tumor tissues have high rates of anaerobic and aerobic glycolysis (32*), and the fact that the acid formed in these processes has been isolated and shown to be d-lactic acid (220, also 103), seem sufficient evidence that tumor tissue is not quantitatively deficient in any of the enzymes required for glycolysis. On the other hand, in the course of the research in which the metabolic pathway of the glycolytic process has been developed, there have been frequent attempts to prove that, in addition to the normal or usual pathway of glycolysis, there is some other pathway which does not include the phosphorylated intermediates. Much of this research (10, 92, 98, 135, 137, 138, 185, 186, 216) has been carried out with extracts prepared from tumor and embryonic tissues and has given the impression that these tissues have a pathway of glycolysis which differs from the normal phosphorylative mechanism. Dorfman (58*) recently reviewed the data which are pertinent to this question and presented a scheme of glycolysis which includes all the intermediates that have been shown to occur in the metabolic pathway which lies between glycogen or glucose and the end product, pyruvic acid. The unanimity of opinion among the workers whose data have gone into the formulation of this scheme leaves no doubt as to what is meant by the term "phosphorylative glycolysis." It is the scheme presented by Dorfman; and it has been presented on various occasions by Meyerhof (141*), by Cori (45*), and by others. It is included in figure 1, in which are presented not only the basic scheme of phosphorylative glycolysis, but all the oxidative and phosphorylative mechanisms which are interlocked with it. These will be discussed in later sections.

In contrast to the precision with which the mechanism for phosphorylative glycolysis has been worked out, the "other" mechanism of glycolysis has never been formulated. The data have been mainly concerned with objections to the phosphorylative pathway rather than with proposals of alternate pathways. Thus, there were the observations that tumor tissues were unable to utilize phosphorylated hexoses at a rate comparable to the glycolysis of glucose (92, 135, 216) and that glycolysis was more rapid in the presence of both glucose and hexose diphosphate than with either alone (98). On the other hand, Boyland, *et al.* (28, 29) found no evidence of summation, and found that the phosphorylated hexoses were converted to lactic acid just as rapidly as glucose. They concluded that "the intermediate processes in glycolysis by tumor tissue are exactly analogous to those of muscle." Their results are undoubtedly due to the fact that they fortified their tumor extracts with coenzyme I and adenylic acid. They called attention to the fact that both compounds are rapidly destroyed by the tumor extracts. This observation is probably the explanation for the results of the previous workers, especially since Ochoa's demonstration (152) of the need for phosphate acceptors in glyclolysis experiments with brain extracts. When the concentration of phosphate acceptors is critical, glucose can be glycolyzed more readily than hexose diphosphate because glucose is a phosphate acceptor as well as a substrate in glycolysis (see Fig. 1). It thus appears that the data constitute further support for the phosphorylating scheme of glycolysis. Tumor extracts may well differ from other tissue extracts, not in the pathway of glycolysis, nor even in the amount of glycolyzing enzymes, but rather in the amounts of certain enzymes which deal with the nonglycolytic reactions of the coenzymes of glycolysis.

The metabolism of any given tissue is the resultant of the balance between the various enzymes that are responsible for each of the reactions in Figure 1. Certain of the starting materials which are stored in the cells or must be brought to the cells are shown at the periphery of Figure 1. These materials include glucose, oxygen, glycogen, fat, and various vitamins which serve as building blocks for coenzymes. The figure does not show the exact mechanisms for the aerobic phosphorylations in the Krebs cycle because these are not known, but each of the di- and tricarboxylic acids shown probably occurs in phosphorylated form. Although inorganic phosphate is probably also esterified in the oxidation of fat (81), present knowledge is too meager to include in the figure. Similarly, the formation of phosphopyruvic acid from the C_4 acids (105) is probable but is not shown.

The other experiments which have been cited in opposition to phosphorylating glycolysis have involved the fact that glyceraldehyde inhibits glycolysis in tumor, brain, and embryo preparations at much lower con-
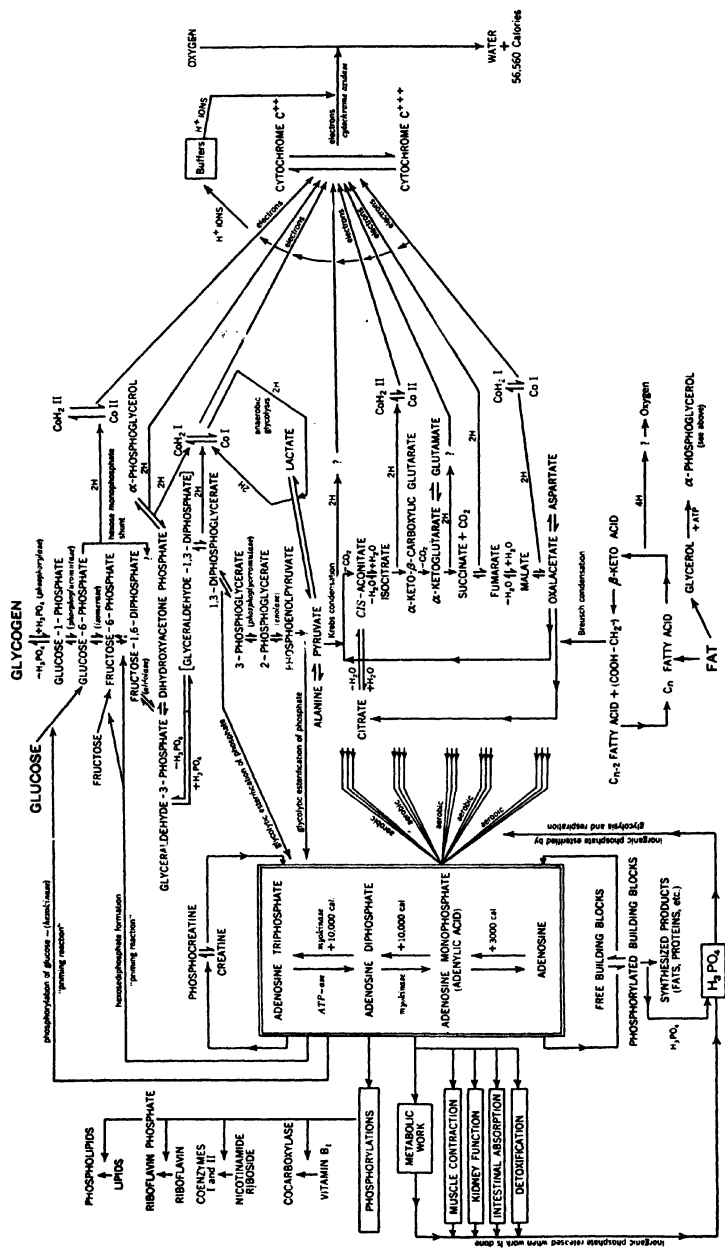
Fig. 1.—Biological energy transformations; the inter-relationships of the phosphorylative, glycolytic, and respiratory enzyme systems. The various areas of the chart, from left to right show: (1) the utilization of phosphate-bond energy, (2) the phosphate-energy reservoirs, (3) the esterification of inorganic phosphate, (4) the glycolytic mechanisms, and (5) the aerobic mechanisms.

centrations than are required to inhibit muscle glycolysis, and glyceraldehyde has been suggested to be a specific inhibitor for nonphosphorylating glycolysis. The many papers on this subject have been reviewed by Dorfman (58*), who conceded the possibility of other glycolytic pathways but pointed out that "the postulation of such alternate pathways is at present unnecessary to explain the available facts." It appears likely that the unique characteristic of uncontrolled growth in tumor tissues is not related to any significant qualitative or quantitative deviation in the enzymes of glycolysis. However, it is entirely possible that there are more subtle changes in the enzymatic make-up of the mechanisms which lead to the formation and utilization of the adenosine phosphate esters, and that these alterations may have been reflected in the results obtained in glycolytic experiments.

2. Oxidative Mechanisms: Metabolic Pathways

Krebs Cycle.—It is apparent from an examination of Figure 1 or the other analogous schemes that have been presented (45*, 58*, 141*, 164*) that the end product of glycolysis is pyruvic acid and that, if oxidation is superimposed upon glycolysis, then lactic acid need never occur as an intermediate inaerobic carbohydrate catabolism, but would occur only in anaerobic glycolysis, or in cases in which the glycolytic mechanism outpaces the oxidative removal of pyruvate. The mechanism of pyruvate oxidation remained obscure until Krebs discovered that fumarate and pyruvate could be converted to succinate aerobically in the presence of malonate, which suggested that the fumarate was converted to succinate by some mechanism other than simple reduction. This led to the concept of a cyclic series of reactions which involved a combination ("Krebs condensation," Fig. 1) between oxalacetic acid and pyruvic acid, followed by a series of oxidations and decarboxylations that ultimately lead to the formation of oxalacetic acid, which is then ready to condense with another molecule of pyruvic acid formed by glycolysis.

The original scheme was referred to as the citric acid cycle; but subsequent work has indicated that citric acid probably is not in the direct path of pyruvate breakdown. The modified scheme therefore eliminates citric acid (115*) as an intermediate in pyruvate oxidation and places greater emphasis on isocitric acid, as we have done in Figure 1, in accord with our 1940 review (159*) as well as with Krebs' 1943 review (115*), which is a thorough discussion of all the data bearing on the theory. In his review, Krebs suggested the name "tricarboxylic acid cycle" instead of "citric acid cycle" for the pathway. The new term, though accurate enough, is rather

unwieldy, and we prefer the term "Krebs cycle," which is both descriptive and convenient. It may be mentioned that isocitric acid dehydrogenase preparations having no activity with respect to citric acid have been prepared by Adler, *et al.* (2); but the converse has never been done, indicating that citric acid is not oxidized as such, but must first be converted to isocitric acid.

That citric acid may be an intermediate in fat metabolism has been recently suggested by Breusch (31). He reported the discovery of a new enzyme (citrogenase) which catalyzes the breakdown of β-keto acids by means of a reaction in which two carbon atoms are removed from the β-keto acid and condensed with oxalacetic to give citric acid and a fatty acid containing two less carbons, as shown in Figure 1. The reaction is given by acetoacetic acid and a number of other β-keto acids. The enzyme occurs in large amounts in muscle, kidney, and brain, but little in liver, and not at all in spleen, pancreas, and lung. Breusch's theory receives considerable support from the work of Munoz and Leloir (145), who were able to oxidize fatty acids, including β-hydroxybutyric acid, by means of a liver enzyme preparation. They found that inorganic phosphate, fumarate, cytochrome c, adenylic acid, and magnesium or manganese were needed for the oxidation, and that the fumarate had to be oxidized. Since fumarate is oxidized to oxalacetate, their work is in harmony with that of Breusch. That citrate is formed is confirmed by experiments by Lardy (117), in which the addition of phospholipids to suspensions of minced rat kidney or heart muscle in the presence of malonate increased the accumulation of citric acid by as much as 20%.

The components of the citric acid cycle are all listed in unphosphorylated form; but there are probably phosphorylated intermediates for every step shown, since Ochoa (153) has reported 15 moles of phosphate esterified per mole of pyruvate oxidized. Kalckar (105) has demonstrated the formation of phosphopyruvic acid during the oxidation of malic acid in the presence of inorganic phosphate, and phosphoenoloxalacetate has been suggested (106*, 122*) as an intermediate in this reaction. This has not been shown in Figure 1, but it must be considered as a probable mechanism not only in this case but in similar esterifications of inorganic phosphate which must occur throughout the Krebs cycle (165).

There seems to be little doubt that the Krebs cycle represents the mechanism of pyruvate oxidation in normal tissues with specialized function, although this has not been proved. On the other hand, there are indications that the Krebs cycle is essentially nonoperative in cancer tissues. As further work is done on the individual enzymes involved, a clearer pic-

ture of the importance of the Krebs cycle in normal and cancer tissues will be obtained.   It is apparent that the usual techniques for demonstrating the Krebs cycle can succeed only in tissues which are rich in coenzymes and relatively poor in factors destroying these coenzymes.   It cannot be said that tumor tissues do not oxidize carbohydrate through the Krebs cycle simply because a technique which gives positive results when applied to pigeon breast muscle gives negative results in the case of tumor tissue, because there are normal tissues which also give negative results under these conditions.   The assay of the tissues for the specific biocatalysts will have to be carried out before the question can be settled.

Hexose Monophosphate Shunt.—The preceding discussion has covered the oxidation of pyruvate.   Since this is the end product of glycolysis, it would seem that sufficient mechanisms for the combustion of glucose are already known.   There is, however, a group of enzymes which are capable of oxidizing glucose-6-phosphate and its oxidation products (55, 56, 76, 120, 221, 222).   This work was done in 1936–1938 and has never been pursued to completion, although it has shown that the breakdown occurs by means of oxidations and decarboxylations.   It has been suggested that the glucose-6-phosphate is oxidized to phosphogluconic acid, which is oxidized to the 2-ketophosphogluconic acid.   It was proved that the next intermediate was a pentose, but this was not positively identified. However, Dickens (55, 56) suggested that d-ribose-5-phosphate was the intermediate because it was capable of further oxidation at a much faster rate than were other pentoses by the yeast preparations with which this work was done.   The hexose monophosphate oxidation has been thought to proceed through the stages of three decarboxylations to form a 3-carbon residue, which could then be metabolized through known pathways. Dickens showed that kidney slices could oxidize 2-keto-d-gluconic acid, which suggests that the hexose monophosphate shunt may occur in animal tissues.   Other evidence for this has been indirect and has been based on experiments in which glycolysis would not occur anaerobically (e. g., tissues poisoned with iodoacetate) but in which glucose could be oxidized under aerobic conditions.   These experiments have been discussed by Shorr (197*).

The evidence for the exact intermediates in this metabolic pathway is so meager that they have not been included in Figure 1.   However, Dickens' suggestion that ribose-5-phosphate is an intermediate in this pathway should be tested, for, if true, this pathway would be expected to assume great importance in growing tissues in which ribose-5-phosphate would be needed as a building block for the synthesis of ribonucleoproteins which have been demonstrated to occur in normal tissues and in various tumors, as well as in the Rous tumor virus particle (48, 52).

Although the evidence is all too meager, there are sufficient data to suggest that the oxidation of glucose may proceed via the hexose monophosphate shunt in growing undifferentiated tissue and in resting specialized tissue, while in the activity metabolism of specialized tissue (114a*, 201*) the Embden-Meyerhof glycolytic pathway and the Krebs oxidative cycle may supervene. According to this view, the hexose monophosphate shunt would represent the pathway which is relatively independent of hormonal influences (197*). The transaminase system (39*) might be highly significant in connection with the mechanisms for gluconeogenesis (see glutamic and aspartic acids, Fig. 1) which, after all, is probably of greatest importance as a means of tapping auxiliary fuel reserves when the carbohydrate reserves are inadequate (200*). The deamination of the glucogenic amino acids leads to the formation of compounds which enter into the Krebs cycle; and, by conversion to phosphopyruvic acid (105) via the C_4 acids, glucogenesis can take place (see Fig. 1). The Lipmann reaction (see page 213) would be especially important in this mechanism.

3. Oxidative Mechanisms: Hydrogen Transport

There has been little advance in the mechanism of hydrogen transpor since the author's review in 1940 (159*). Figure 1 shows the steps in the transport of hydrogen from the substrates to oxygen as far as these are known at present. The existence of a direct connection between coenzyme II and cytochrome c is based upon the work of Haas, et al. (89, 90), although their work was done with yeast, and no clear-cut demonstration of this reaction in animal tissues has appeared. In the case of the oxidation of pyruvate and α-ketoglutarate, still less is known regarding the pathway of hydrogen transport. Experiments by Schneider and Potter (191) seem to indicate that coenzyme I may be directly involved in the oxidation of pyruvate. Coenzyme I is definitely oxidized via cytochrome c. Experiments by Potter and Albaum (167) and by Straub (207) have reaffirmed the observation (160) that succinic dehydrogenase activity is unnecessary for this oxidation.

Inhibitor studies with the succinic dehydrogenase system (169) led to the suggestion that this enzyme possessed an activating center for succinate which was distinct from the activating center for cytochrome c. This indicated the probability of electron transmission between the two centers. Further support for the concept has been obtained from kinetic studies by Atkin (4), which the author called to our attention. It was demonstrated that malonate interferes with the donor:enzyme function but not with the

enzyme:acceptor function, which were therefore considered to be located at different points on the enzyme.

4. Phosphorylative Mechanisms

Priming Reactions.—These reactions include the phosphorylation of glucose to glucose-6-phosphate by means of the enzyme, hexokinase, which catalyzes the reaction between glucose and adenosine triphosphate (ATP) (140, 152). It is referred to as a priming reaction because it represents an expenditure of ATP energy in order to activate a substrate which then undergoes a series of reactions resulting in the esterification of more than enough phosphate to repay the phosphate debt incurred in the priming reaction. In addition to the hexokinase reaction, there is another phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate (105), and fructose can also be converted to fructose-6-phosphate (45*, 105). The metabolism of glycogen requires the expenditure of less esterified phosphate than does the metabolism of glucose, because the latter uses up ATP in getting to the glucose-6-phosphate level, whereas glycogen does not. Thus, a phosphate debt of two moles of phosphate is incurred per mole of glucose, while a debt of only one mole of phosphate is incurred per mole of hexose from glycogen. Lipmann (122*) has suggested, on the basis of available evidence (93, 128), that glycogen may be converted to fructose 1,6-diphosphate, without using ATP phosphate, by means of glucose formation. This reaction would be as follows:

Fructose-6-phosphate + glucose-6-phosphate ⇌ fructose-1,6-diphosphate + glucose
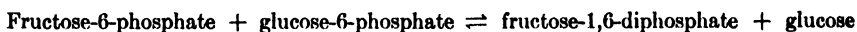
This hypothetical reaction is of considerable importance *per se*, and also in view of the suggestion that normal liver glycolysis is based on glycogen rather than glucose breakdown, whereas hepatomas will glycolyze glucose (57, 156, 180). The reports indicating high anaerobic glycolysis of glycogen in normal livers have, however, been suggested by Burk (33) to be atypical. An ultimate answer as to whether normal liver differs from hepatomas in this part of the glycolytic scheme will have to wait until specific assay methods for hexokinase, phosphoglucomutase, phosphorylase, isomerase, and the additional unnamed enzymes have been worked out and applied.

An additional priming reaction is the conversion of glycerol, which results from the breakdown of fat, into α-phosphoglycerol using the phosphorylating capacity of the ATP system (105). This compound could then be metabolized further through the carbohydrate pathway, as shown

in Figure 1, by oxidation to glyceraldehyde phosphate. The reverse reaction is probably important in the conversion of carbohydrate to fat.

ATP Synthesis.—The mechanism by which ATP is resynthesized by anaerobic processes is well known. It is shown in detail in Figure 1, and has been amply discussed elsewhere (45*, 106*, 122*, 141*). Of the two steps which lead to ATP synthesis, the reaction between phosphopyruvic and adenylic acids was the first to be recognized, yet only recently strong evidence has appeared which indicates that this reaction is dependent upon potassium ions (25, 26).

The remaining phosphate esterification which occurs in glycolysis namely, the oxidative step, was formerly referred to as triose phosphate (*i. e.*, glyceraldehyde-3-phosphate) oxidation. As Kalckar has pointed out (106*), "The clarification of the coupling between the triose phosphate oxidation and the uptake of inorganic phosphate represents one of the greatest advances in modern biology." The Warburg school succeeded in showing that the product of this oxidation is 1,3-diphosphoglyceric acid (151) and that it involves the esterification of inorganic phosphate (224). It was assumed by the authors and by most workers in the field that 1,3-diphosphoglyceraldehyde is formed nonenzymatically as the substrate for the oxidative reaction. However, recent work by Meyerhof and Junowicz-Kocholaty (142) strongly suggests that the hypothetical 1,3-diphosphoglyceraldehyde is nonexistent, and that the monophosphate and inorganic phosphate are simultaneously adsorbed on the triose phosphate dehydrogenase. In removing one hydrogen from the aldehyde, and one from the phosphate, the enzyme effects the formation of the 1,3-diphosphoglyceric acid. If this is indeed the mechanism of formation of this particular high-energy phosphate bond, and of analogous bonds in other metabolites, the isolation of the phosphorylated precursors may not be possible. The mechanism is important because it is probably followed throughout the Krebs cycle of oxidations. No matter what the detailed mechanism is, the main point is that inorganic phosphate is converted to a high-energy phosphate bond by means of an oxidative reaction. With the demonstration of this important fact, it was possible to see the unifying principle which underlies vital processes and to begin the integration of enzymatic reactions, so necessary to an understanding of vital phenomena.

The demonstration of phosphate esterification in oxidative processes followed quickly upon Warburg's discovery in 1939. Kalckar had in 1937 (104) observed phosphorylation in kidney extracts under aerobic conditions and shown that phosphorylation was proportional to oxygen uptake. He later also showed (105) that numerous compounds could be

phosphorylated via the ATP system, which was maintained by the oxidations.   Kalckar obtained phosphopyruvic acid as the product of malic acid oxidation.   Lipmann (122*) has suggested that this arises from phosphoenoloxalacetate formed by the oxidation of malate and inorganic phosphate. This is not shown in Figure 1, but it should be regarded as a likely step in the conversion of the dicarboxylic acids to glycogen, as well as in the phosphorylation mechanism.   Belitser and Tsybakova (18) showed that inorganic phosphate could be esterified by the oxidation of lactate, pyruvate, and the 4-carbon dicarboxylic acids, and that this phosphate could be stored as phosphocreatine.   They obtained two moles of phosphate esterified per atom of oxygen taken up.   Ochoa (154) has demonstrated the esterification of inorganic phosphate coupled with the oxidation of α-ketoglutarate to succinate, the further oxidation of which was blocked by malonate. Cori and coworkers (41, 43, 44) made further studies on the oxidative phosphorylations in kidney extract and found (41) that ten moles of phosphate were taken up per mole of glucose burned.   More recently Ochoa has reported (153) three moles of phosphate esterified per atom of oxygen taken up, that is, 15 moles of phosphate per pyruvate burned (see Fig. 1), which would be equivalent to 36 moles of phosphate esterified per glucose burned. This was on the basis of correcting for the inorganic phosphate which escaped back into the system through the enzymatic breakdown of ATP. If these data are correct, it is apparent that the oxidation of the original substrate is not the only reaction in which inorganic phosphate is taken up, and that one must look for other phosphorylations either in connection with a modified Szent-Györgyi hydrogen transport scheme, or in the oxidation of the reduced coenzymes and of cytochrome c (18, 41, 106*, 122*).

It is this fact which makes the work of Meyerhof and Junowicz-Kocholaty so important, since, in seeking the mechanism of oxidative phosphorylation according to the Warburg explanation, we have been restricted to reactions in which a spontaneous esterification of the substrate and phosphate could occur.   According to the Meyerhof mechanism, we apparently need only a group which can release one electron because the other electron is furnished by the phosphoric acid.   The pH optimum of these reactions may indicate that the reacting phosphate is the divalent anion $(HPO_4)^{--}$. Umbreit has pointed out (217*) that the esterification of inorganic phosphate during the oxidation of sulfur by *Thiobacillus thiooxidans* (218, 219) may provide a model for the esterification of phosphate coupled with electron transfer.   The demonstration (41) that ten moles of phosphate are esterified in addition to that required for the priming reactions (see above) implies that the energy of glycolysis and respiration captured by the phos-

phorylating mechanism is used for something in addition to the priming reactions. The utilization of ATP energy will be discussed below.

ATP Utilization.—In the paper cited above (41) it was pointed out that, in the intact cell, "the energy derived from oxidations instead of being used exclusively for the transfer of phosphate to glucose is diverted to a variety of other cellular functions." Kalckar (106*) and Lipmann (122*) have both emphasized this concept, and have discussed the various specific types of cellular function for which evidence is available. Figure 1 is merely a graphic representation of these views, presented in such a way as to show how the various systems are interrelated. It is not the purpose of this review to cite all the papers which bear on the validity of this concept. In the case of the synthesis of fat, protein, and other organic compounds, one would not expect the phosphate energy to have to go through the adenylic system in order to be used, if the building blocks occur in the phosphorylated state in the course of metabolism; but even in this case the phosphorylated building blocks are probably in equilibrium with the ATP system and the synthesis is dependent upon the energy level of the system. The synthesis of glycogen from glucose has been taken as a model for the other syntheses; in this instance the process may be traced in detail in Figure 1, and has been very elegantly performed experimentally by Colowick and Sutherland (42). These workers were able to effect the synthesis of glycogen from glucose, using ATP as the source of energy, and barium ions to remove the inorganic phosphate formed in the final step. In the isolated metabolic sequence, there was a quantitative relation as follows:

$$n \text{ ATP} + n \text{ glucose} \longrightarrow \text{polysaccharide, } i. \ e., \ (\text{glucose})n + n \text{ ADP} + n \text{ phosphate}$$
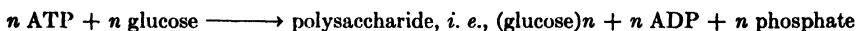
They pointed out that *in vivo* the inorganic phosphate is removed by being esterified to ATP, which thus provides for both the continuous removal of inorganic phosphate and the formation of ATP (see Fig. 1).

It is easy to see how phosphate energy can be utilized for endergonic syntheses, and even for processes like kidney function and intestinal absorption, where the processes are in the nature of osmotic work against a concentration gradient (106*, 122*). But in the case of muscle contraction, the work is mechanical and the biochemical nature of the process was completely unknown until very recently, despite the fact that it has been apparent for some time that ATP was the source of energy for muscular work. The relation between the biochemical and physiological aspects of muscular contraction has become somewhat clearer as a result of the brilliant researches of Engelhardt (75*) and coworkers, who have shown that ATP-ase is either identical with or intimately connected to myosin, the contractile

element of muscle.   Their observation that myosin and ATP-ase could not be separated has received wide confirmation (8, 149, 150).   It was further shown that, in the presence of ATP, artificially prepared myosin threads exhibited a characteristic lengthening which did not occur in the presence of any other substance tried, including ADP and adenylic acid.   From this it might be inferred that the myosin fiber is in the relaxed form when it is saturated with ATP in the sense of an enzyme being saturated with its substrate.   Contraction would then result from the conversion of ATP to ADP and would coincide with the liberation of inorganic phosphate.

There has been considerable opposition to the application of studies on tissue extracts to *in vivo* processes.   Sacks (183*, 184) in particular has denied that ATP dephosphorylation furnishes the energy for muscular contraction.   His studies with radioactive phosphorus in intact animals, which seemed at variance with modern theories of energy transfer, now appear to have been greatly complicated by the failure to differentiate between intracellular inorganic phosphorus and extracellular inorganic phosphorus.   Furchgott and Shorr (81) have recently demonstrated that, when this distinction is made, the results show that the *in vivo* observations are completely in harmony with the *in vitro* predictions.

In view of what is known about the formation of ATP in tissues other than muscle, it is of considerable importance to ascertain the form and function of ATP-ase in these tissues.   Obviously myosin as such does not occur in the other tissues; but it seems possible that myosin is the specialized form of ATP-ase which is peculiar to muscle, while other tissues may contain ATP-ase modified according to the specific functions of the respective tissues.   It is perhaps significant that proteins which belong to the keratin–myosin group on the basis of x-ray studies have been reported in kidney (12), and that fibrinogen and fibrin also appear to be members of this group (9).   It will be recalled that the clotting of blood is a calcium-activated reaction.   Heilbrunn (94*) has emphasized that this calcium-activated clotting phenomenon is a property of protoplasm in general, which may indicate a fundamental unity among the ATP-ase proteins of tissue other than muscle, since ATP-ase is a calcium-activated enzyme (8, 63).   It is not possible at present to discuss the possible form and manner of function of this protein in tissues other than muscle; but whatever this may prove to be, DuBois and Potter (63) have been able to demonstrate the presence of ATP-ase in a wide variety of normal tissues.   Proof that the ATP-ase in these tissues is related to function has not been possible as in the case of muscle, where the contractile function is obvious and has received the closest scrutiny for many years.   However, DuBois and Potter

(62) reported the activation of ATP-ase in the submaxillary gland by means of acetylcholine, which is known to stimulate the gland to function. Potter and Liebl (171*) have also shown a marked increase in the ATP-ase content of tissues of new-born rats, during the period when the tissues are assuming a functional load not present in the embryo (166*). ATP-ase determinations on rat embryos showed that the ATP-ase content did not vary in the period immediately before and after birth. These observations do not prove that ATP-ase is responsible for the function of the various tissues, but they certainly indicate the possibility. It is hoped that experiments can be devised to test the relationship more directly.

5. Control Mechanisms

One of the most important problems which confronts the investigator in the field of cancer research is the problem of control, since the absence of control is the outstanding feature of cancer metabolism (see page 228, *et seq.*). Until recently, there has been no foundation on which to build an understanding of the uncontrolled growth of cancer tissue because the mechanisms for the control of normal metabolic processes remained unknown. It is true that a great deal is known from the physiological standpoint, but this is largely in terms of the outwardly visible aspects of organic function and the relationship of one organ to another. In the case of cellular metabolism, however, relatively little progress has been made in terms of control mechanisms. Yet with the cancer problem it is precisely in this field of cellular metabolism that the ultimate answer must be found, since the growth impulse in cancer tissue comes from within the cells themselves.

Cells contain, in general, certain reservoirs of energy, which include phosphocreatine, adenosine triphosphate, glycogen, and fat. Also present are the enzymes needed for the breakdown of these substances. It is well known that the rate of breakdown of these substances is related to the activity of the cells in question and that these rates may vary over a wide range. In a yeast cell, or in a cancer cell, there is no particular need for control. The sole activity is growth, and energy-yielding substrates may be catabolized just as rapidly as building blocks for growth can be made available. In differentiated tissues with specialized functions the picture is entirely different. Periods of intense activity are interspersed with periods of relative quiescence or at least basal metabolic rates.

These specialized tissues differ in at least three important respects from primitive, growing, nonspecialized tissues. First, the tissue must be equipped to hold energy reservoirs intact until the functional demand occurs, at which time the reservoirs must be mobilized rapidly to provide

energy for the function.   Second, the remainder of the organism must be
regulated so as not to interfere with the needs of the functioning part.
Third, there must be a mechanism by which the specialized organ can
grow and develop to meet functional demands which may temporarily
tax its capacity.   The first point has been investigated in terms of enzymes
and intermediate metabolism.   The second point represents the contribu-
tion of physiologists, endocrinologists, and pharmacologists.   The third
point is perhaps of greatest interest in connection with the cancer problem,
in which growth appears to occur in the absence of a continuing stimulus
(see the fourth cancer postulate, page 202).   These three steps in the re-
sponse of a specialized tissue to a stimulus are in reality control mechanisms,
and must be finally correlated with each other and with the original stimu-
lus through common denominators.   It is here proposed that the adenosine
triphosphate system may be just such a common denominator, which will
serve to integrate the above three control mechanisms to be discussed in
separate sections below.   Before proceeding to these points, however, it
must first be shown that the adenosine triphosphate system is related to
the stimulus and the functional response.

It is apparent from the data available that the adenosine triphosphate
system (enclosed in a box in Figure 1) is a common denominator by which
all of the reactions of glycolysis and respiration can be made to pool their
respective energies in one compound.   This correlation alone makes the
ATP system of paramount interest as a possible connecting link between
function and the energy-yielding reactions.   In order to buttress this
correlation, it can be shown:

    (a) that the breakdown of ATP is correlated with function,
    (b) that ATP breakdown results from the stimulus, and
    (c) that it leads to the occurrence of glycolysis and respiration.

The first point has been confused by the fact that the ATP energy reservoir
is in dynamic equilibrium with the other sources of energy and is not de-
pleted under normal energy demands.   Indeed, if ATP is the energy reser-
voir which is directly connected to function, and if all other energy sources
are connected to function via this compound, then it is essential that the
refilling mechanisms be able to keep pace with the depleting mechanisms.

The literature on this point need not be fully documented here but the
following papers may be cited.   Lundsgaard (127) showed that frog
muscles poisoned with iodoacetic acid could contract anaerobically without
the formation of lactic acid, at the expense of phosphocreatine.   He also
found that, in these preparations, ATP did not break down until after the

onset of rigor (128).   Flock, Ingle, and Bollman (80) showed that, when severe work demands were placed upon the leg muscle of surviving rats, ATP breakdown did not occur to any extent for 40 seconds, while other changes during this period were quite pronounced (see Fig. 2).   With regard to point (b), the most significant fact which has recently come to light is Bailey's finding (8) that calcium acts as a coenzyme for ATP-ase in the myosin system.   That ATP-ase in tissues other than muscle is also activated by calcium has been demonstrated by DuBois and Potter (63).
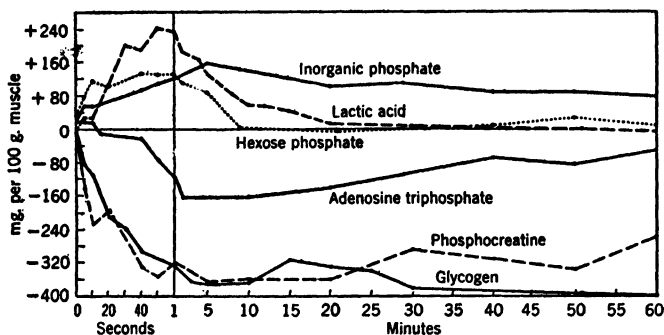
Fig. 2.—Changes in the concentration of muscle metabolites in the initial and in the continuous phases of muscle contraction with high work output.   Rat leg muscle was stimulated three times per second with a 100-gram load.   A shift from anaerobic to aerobic metabolism occurs in about one minute.   (Flock, Ingle, and Bollman, 80.)

That the release of calcium is an accompaniment of stimulation in a wide variety of cells has been the theme of a large number of publications by Heilbrunn and his students.   Heilbrunn's book (94*) is filled with examples of the calcium mechanism.   A concise statement of the Heilbrunn theory is given in the following words (136): "A primary effect of a stimulating agent on a cell is to cause a release of calcium from organic combinations in the cortex of the cell into the main body of protoplasm."   This organic complex may include ascorbic acid (173).   Mazia showed that the fertilization of the sea urchin egg was followed by a great increase in diffusible calcium.   Similar studies were carried out with muscle tissue (95, 228). The activation of the ATP-ase system in submaxillary gland by acetylcholine (62) was obtained in the absence of added calcium; when an excess of calcium was present, acetylcholine produced no further activation.

Bailey (8) suggested that the calcium ion might be the means by which the stimulus is able to increase the rate of ATP breakdown in muscle. It is not yet clear whether sodium ions will permit a slow rate of breakdown which is merely accelerated by calcium ions, but none of Bailey's active myosin preparations were devoid of ATP-ase activity even when calcium was not added. The proposed relation between the stimulus, calcium, ATP breakdown, and function is shown in Figure 3, in the upper third of the figure. We may now turn to a consideration of the three control mechanisms which are placed in operation when a stimulus causes ATP breakdown in a particular tissue.

Direct Control.—It was stated above that the specialized tissue must be equipped to hold energy reservoirs intact until the functional demand occurs, and that function is correlated with ATP breakdown. It has been shown that ATP breakdown may be controlled by the maintenance of calcium ions in an undissociated form until a stimulus reaches the cells. It remains to be shown that the combustion of the carbohydrate energy reservoirs does not occur unless preceded by ATP breakdown: this inherent, direct, or intracellular control is a result of the fact that glycolysis cannot occur in the absence of the breakdown products of ATP, plus the fact that the processes of glycolysis and respiration remove the products of ATP breakdown from the system and convert them back to ATP.

Belitser (18a) appears to be the first to attempt a formal statement of the concept, although he traces the literature back to Engelhardt's reference to phosphagen in 1932. Belitser attempted to explain the regulation of muscle respiration in terms of the availability of free inorganic phosphate and phosphate acceptors, thus: "À part l'acide lactique, le phosphagéne, dont les produits de clivage stimulent les processus d'oxydation, joue un rôle important dans la régulation de la respiration du tissu musculaire. Ainsi, le ralentissement graduel de la respiration du muscle isolé, au début de l'aerobiose, s'explique evidemment par la synthèse du phosphagène qui diminue la teneur du muscle en créatine et en phosphates libres." Johnson (100*) extended the idea to the problem of the mechanism by which respiration decreases glycolysis, that is, the Pasteur effect. He proposed that the regulation of glycolysis by respiration could be explained if the concentration of inorganic phosphate required for maximum glycolysis is higher than that required for maximum respiration, and cited data which suggest that this is the case. Lipmann (122a*) has questioned the value of these schemes because "they disregard controlling factors in the cell which undoubtedly must regulate the routes of phosphate turnover." On the other hand, we take the position that the inherent intracellular control which is the basis of life in primitive cells, is also the foundation upon which the specialized mechanisms must be laid. The "controlling factors in the cell" must arise in response to metabolic events within the cell or in other cells. In the latter case, pharmacological mechanisms are most certainly involved, and will be briefly mentioned later.

These mechanisms must be superimposed upon the basic mechanism of intracellular control, which accordingly should be examined in some detail. The mechanism of direct control is shown in Figure 3, but the details, so far as they are available, must be found in Figure 1. The very first reaction in glycolysis, namely, the conversion of glycogen to glucose-1-phosphate, does not occur in the absence of inorganic phosphate. Indeed, whenever the ratio of inorganic phosphate to glucose-1-phosphate falls below a value of 3 to 4 (depending on the pH), glycogen splitting ceases and glycogen synthesis occurs (46).

Figure 3 emphasizes that the breakdown of ATP is not catalyzed by the same enzymes which lead to its resynthesis, and that the two processes occur simultaneously at widely varying rates. (The detailed mechanisms by which glycolysis and respiration are controlled, as well as the means by which they lead to the resynthesis of ATP are shown on Figure 1.) The organismic aspect of the pharmacological control is not detailed here, but it can be integrated with the action of the autonomic nervous system (see Gellhorn, 82*). The significance of these relationships to growth and to the cancer problem is indicated by showing probable sites of carcinogenic and cocarcinogenic action. It is also emphasized that injuries, irritations, and cocarcinogenic procedures in general may all tend to shift the key reaction to the right not only by accelerating ATP breakdown but also by retarding ATP resynthesis.

It is apparent that a continuing breakdown of ATP will lead to glycogen splitting and that, if ATP breakdown stops, glycolysis will stop soon afterward. Inorganic phosphate is also required for the oxidation of triose phosphate, a · shown by Warburg and Christian (224). Thus, on the basis of these two reactions alone, glycolysis of both glucose and glycogen can be regulated by means of ATP breakdown. Although the operation of the Krebs cycle could be regulated indirectly through the glycolytic system, since the end product of glycolysis is the substrate for the Krebs cycle, it appears quite certain that the oxidations of the cycle require inorganic phosphate also, but this is more difficult to demonstrate due to the presence of ATP-ase in the systems which perform these oxidations. Lipmann (121*) showed that inorganic phosphate is required for pyruvate oxidation in a partially isolated system, and Ochoa (154) has recently shown that inorganic phosphate is a limiting factor in the oxidation of α-ketoglutarate.

Not only inorganic phosphate but also adenylic acid regulates glycolysis and respiration. In this case, the function is that of a phosphate acceptor. The key reactions in the glycolytic mechanism are the conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid (151, 224) and the conversion of phosphoenolpyruvic acid to pyruvic acid (124) as shown in Figure 1. Adenylic acid is involved in the aerobic reactions also, as Ochoa has pointed out (153, 154). Recent studies indicate that ADP may be a more effective

phosphate acceptor in the case of both glycolysis (26) and respiration (154). The demonstration (81) that the per cent of radioactive phosphorus in the terminal phosphorus of ATP is identical with the per cent of radioactive phosphorus in the inorganic phosphate of the tissue while the second phosphorus has a much lower radioactivity is further indication that ADP may play a prominent role as phosphate acceptor. If certain systems use one or the other phosphate acceptor preferentially, the sensitivity of control could thus be increased.
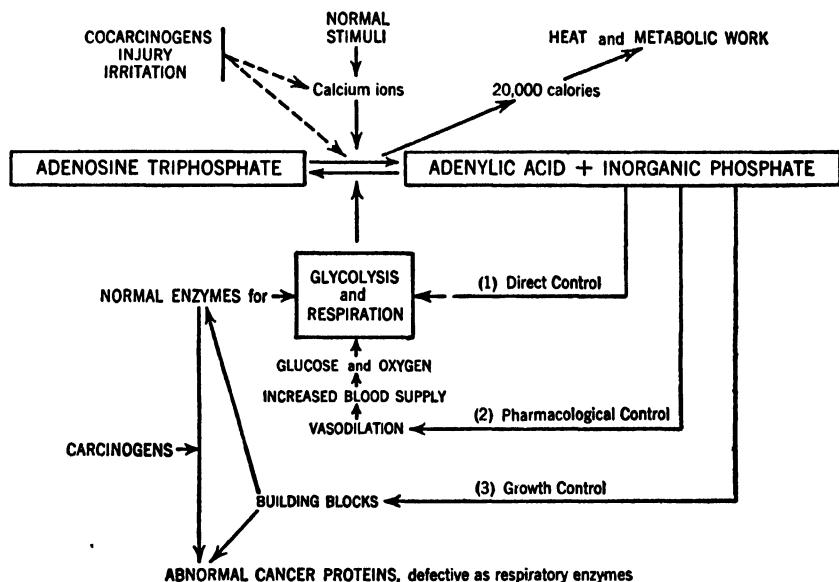
Fig. 3.—Possible mechanisms for the control of cellular and organismic physiology through the integration of the stimulus and the response with the enzymes of glycolysis and respiration, using the adenosinetriphosphate system as the connecting link, or common denominator.

The net effect of all these relationships is that the tissue conserves its glycogen until ATP is broken down, and then only breaks down enough glycogen to resynthesize the ATP.   It is of course implicit in this discussion that the regulation of fat and protein metabolism is also a function of the same control mechanism.   It must also be recognized that there is a basal rate of ATP breakdown which is balanced by the resting metabolism, and that glycogen may be expected to break down only when there is a sudden shift in the ATP/ADP balance.

Pharmacological Control.—The second requirement of a specialized tissue is that, when it is thrown into a state of intense activity, its needs must be integrated with those of the rest of the body. The control mechanism in this case may conveniently be referred to as pharmacological control.

When a tissue is stimulated to increased activity, the flow of blood through the tissue increases proportionately. This phenomenon has been referred to by physiologists as the "nutritive reflex" and is understood to be due to metabolites from the tissue (82*). The metabolites produce local vasodilatation and also stimulate the sympathoadrenal system which causes a vasoconstriction in the nonfunctioning parts of the organism but not in the working tissue (82*), thus subordinating the rest of the body to the needs of the working part. If the work is widespread, the cardiac output increases in accordance with the increased venous return, which is a result of vasodilatation. One of the most likely substances responsible for this hyperemia is adenylic acid (adenosine monophosphate) or adenosine, which result from ATP breakdown. Although lactic acid and carbon dioxide, which are formed concomitantly with ATP breakdown, may play a part in the phenomenon (57a), adenosine is itself a powerful vasodilator (60*); Rigler (176) has reported vasodilatation in working muscles in which the formation of lactic acid and carbon dioxide was prevented by iodoacetate, which does not interfere with ATP breakdown (80, 127, 129).

It is well known that the course of metabolism in the working muscle is different during the first minute of work than it is subsequently when the blood supply becomes established. This fact has been elegantly demonstrated by Flock, Ingle, and Bollman (80), whose data appear in Figure 2, referred to earlier. During the first minute of muscular work, lactic acid, inorganic phosphate, and hexose phosphates accumulated, and ATP, phosphocreatin, and glycogen decreased. As soon as the blood supply became increased to the working level, lactic acid and hexose phosphates began to return to the resting level, and ATP began to accumulate, while inorganic phosphate decreased somewhat and approached a steady state.

It thus appears that the first response of a tissue to a stimulus is the breakdown of ATP. This calls into play the intracellular or direct control mechanism which regulates glycolysis and respiration, which attempt to maintain ATP. In the face of a continuing demand, the cells' local carbohydrate reserves are inadequate and the blood supply must be increased to bring in glucose as a substrate for glycolysis and oxygen in order to burn the products of glycolysis. It appears that the ATP system is closely concerned both with the direct control and with the pharmacological control

which regulates the blood supply.   These relations are shown in Figure 3.

There are other pharmacological controls which are superimposed on the inherent control of the individual cells.   In addition to the "nutritive reflex" referred to above, tissue activity sets in motion a sequence of metabolic events which eventually lead to an increased supply of stimulating hormones (adrenalin, insulin) and neurohumoral factors (sympathin, acetylcholine) to the tissues.   These factors probably alter the basic metabolic pattern so as to increase the speed with which the *status quo ante* may be restored.   Although they may lead to new reaction equilibria it is likely that their ultimate effect may be expressed in terms of the inherent control mechanism of the cells upon which they act.

Growth Control.—In the opinion of the reviewer, the integration of ATP breakdown with the direct control of glycolysis and with the pharmacologic control is sufficiently developed from an experimental standpoint to be regarded as highly probable.   There remains to be considered the third control mechanism, which is called into play in the case of a specialized tissue, namely, that of growth in response to need.

In discussing growth, one must recognize that it can occur in two different ways or by a combination of the two.   There may be *simple hypertrophy*, which is defined as an increase in the size of the cells in the tissue (54), and which is "usually associated with some increased functional demand upon the cells and an increase in their functional capacity."   In addition to hypertrophy, growth may be due to *hyperplasia*, in which cellular proliferation or increase in numbers of cells is occurring (54).   When a tissue grows by hypertrophy it is probably the specialized cells which increase in size;   and when hyperplasia occurs, the source of new growth is probably the undifferentiated cells, which are present to some extent in practically every tissue (54, 175).   It is generally believed that "the acquirement by certain cells of special functional powers as a result of the physiological division of labor has involved the impairment of some of their more primitive general capacities, among these that of reproduction" (54, also 175).   One of the problems in understanding growth is the mechanism by which the undifferentiated cells are held in check in tissues which are not growing.   We prefer to assume for the present that the same factors which make possible the hypertrophy of specialized cells, lead to hyperplasia in the undifferentiated cells.   In the following discussion, therefore, the word "growth" will be used without distinguishing between hypertrophy and hyperplasia.   We may now consider what factors may be responsible for the control of growth, how these factors may operate, and how they are related to the energy-controlling mechanisms.

It is apparent that growth is a self-limiting process in specialized tissues, and that the control of growth is not the simple obvious mechanism which occurs either when a growth factor is added to a bacterial culture medium that is deficient in the factor or when a vitamin is added to a vitamin-deficient diet. Yet, in these instances, it can be proved that the organism is unable to grow when only one extremely minute component of its final protoplasm is missing from its nutrient intake. It is not unreasonable to suppose that, in the case of the self-limiting growth process (in which growth occurs and ceases in accordance with the needs of the specialized tissues independently of the fact that the nutrient intake is not deficient in any factor), control can be effected by means of a self-imposed deficiency of a growth factor. This growth factor would have to be present in relatively low concentrations when the tissue is able to cope with the demands placed upon it, and in larger concentration when the tissue is reaching the limits of its capacity for performance. Both inorganic phosphate and adenylic acid are possible self-limiting growth factors in this connection, since they are present in very low concentration in resting specialized tissues, and their concentration rises and falls in accordance with the balance between the demand placed on the tissue and the ability of the tissue to mobilize energy to meet the demand, as in Figure 2.

According to this mechanism, which is shown in Figure 3, the specialized cells prevent their own hypertrophy under ordinary circumstances by a self-imposed deficiency of strategic building blocks which are also integral parts of the energy-transferring mechanism. This same mechanism could logically be used to control the growth of the undifferentiated cells.

Although there is no direct proof that one or both of the breakdown products of ATP represent the key compound by which a self-imposed deficiency is able to regulate growth, there is an abundance of evidence to show that these two compounds are ubiquitous constituents of compounds which are obligatory parts of protoplasm. Both coenzymes I and II (187*), as well as the flavin–adenine dinucleotide of Warburg and Christian (223), contain both adenosine and phosphate. In these compounds the phosphate is present in both ester and pyrophosphate linkage. Although the synthesis of the latter linkage undoubtedly requires an energy source, the ester linkage is no different from the linkage of any other ester derived from an acid and an alcohol, and as such is in equilibrium with inorganic phosphate (122*).

Similar considerations apply to cocarboxylase, which is the pyrophosphate of vitamin B_1. The monophosphate is a simple ester linkage and is therefore probably in equilibrium with inorganic phosphate. The addition

of the second mole of phosphate involves the formation of a pyrophosphate, or high-energy phosphate bond, and therefore requires an energy source. This second reaction may displace the ester equilibrium away from the free vitamin and inorganic phosphate.

One of the most important uses of adenylic acid and inorganic phosphate from the growth standpoint is the formation of nucleic acids. The nuclear nucleic acids or desoxyribose type do not, of course, contain adenylic acid; but large amounts of phosphate enter into their structure, and they may be derived from the ribonucleic acids (see page 231). In the case of the cytoplasmic nucleoproteins, which are of the ribose type, both adenylic acid and phosphate enter into the structures; either compound could conceivably be used to regulate the synthesis of these substances and, therefore, growth.

In all of the compounds which contain adenylic acid, there is the possibility that adenylic acid as such is not the building block, but that ADP or ATP is actually the molecule which reacts with the other components of the final product. On this basis, the relation between growth and need would not be mediated by adenylic acid but by inorganic phosphate. There are, of course, a number of concomitant reactions which occur when ATP breaks down, and end results of this action include not only inorganic phosphate and adenylic acid but also changes in pH and probably in oxidation-reduction potential, together with the formation of carbon dioxide as well as lactic acid and other metabolites. The present discussion is intended only to call attention to the need for correlating these phenomena with the self-limiting growth process, and to point out that inorganic phosphate and adenylic acid are more obviously connected with the self-limiting growth process than any other factors which can be called to mind. It may be recalled that the so-called wound hormone which Loofbourow and associates have studied has recently been shown to be similar to or identical with the adenosine phosphates (125). Another paper of interest in this connection is that of Visscher and associates (196) who studied phosphocreatine and glycogen levels in the heart muscle of rats in which cardiac hypertrophy had been produced by means of feeding a thyroid preparation. They found that the levels of phosphocreatine dropped from normal values of about 12 to values of 5–6 mg. of phosphocreatine phosphorus per 100 g. of muscle, while the glycogen values dropped from around 500 mg. to values around 100 mg. per 100 g. of muscle. These workers did not measure ATP or inorganic phosphate; but if the relationship shown in Figure 2 is similar in cardiac and in skeletal muscle, one might infer that the level of inorganic phosphate was increased in the hearts undergoing hypertrophy.

A Theory of Cancer.—On the basis of the theory that growth in a specialized tissue represents a response to a need, while self-limitation of growth is brought about by the removal of certain essential building blocks, it is perhaps worth while to see if this concept can be applied to the cancer problem.   Such an attempt has been made by the author (163) in presenting a theory of cancer consisting of nine main points, which were essentially as follows:

1.   Cancer is considered to result from the introduction of an abnormal protein (which is called a "cancer virus"; though most cancers have given only negative evidence (147) of being caused by a virus in the strict sense, it seems quite likely that an altered protein is present in cancer tissue (79*, 119); obviously this protein would have to be isolated and shown to transmit the characteristic malignancy before it could be called a true virus).   The altered protein is assumed to arise spontaneously in certain cases, to be formed by the continued action of carcinogenic agents in other cases, and to be introduced into the cell from other infected cells in the case of tumors known to be of virus origin.

2.   The "cancer virus" is assumed to be almost identical with an enzyme X, except that it lacks the specific catalytic potency of this enzyme.   Enzyme X is considered to be a constituent of normal differentiated cells and on the basis of present data is thought to be a complex of respiratory enzymes, possibly of the nature of a ribonucleoprotein complex.

3.   Both the "cancer virus" and enzyme X are assumed to require essentially the same building blocks for their synthesis; and the characteristics of carcinogenesis may be explained in large part on the basis of competition between the "cancer virus" and enzyme X for the building blocks.   The cancer-inhibiting effect of various dietary restrictions (182*) might be explained on the basis of this competition.

4.   The synthesis of both enzyme X and the "cancer virus" is assumed to proceed autosynthetically.   Thus, the results of the competition between the two proteins are assumed to depend upon their relative concentrations.

5.   On the basis of the above considerations, it would follow that a "cancer cell" would result whenever the ratio of "cancer virus" to enzyme X attains a value such that the synthesis of enzyme X could no longer successfully compete for the building blocks. As the amount of the "cancer virus" increases, it competes more and more successfully for the building blocks, until the restraining competition of enzyme X is thrown off completely.   This explanation accounts for the sudden appearance of cancer (the fifth postulate, page 202) and also for Blum's theory (24) that "normal tissue exerts a controlling influence on tumor development which becomes less as the tumor grows, until finally the tumor virtually escapes from this influence and has almost unrestricted growth for a time."

6.   It is assumed that both enzyme X and "cancer virus" are broken down in time, with antigen–antibody reactions playing an important role in the case of the latter.   If the disposal of the "cancer virus" is rapid enough, the competition with the normal enzyme is unsuccessful and no cancer cells result.

7.   According to the above concepts, a *carcinogen* would be any agent that would convert enzyme X into "cancer virus," and a *cocarcinogen*, which is an agent that alone cannot produce cancer (20, 21, 195), would facilitate cancer production either by in-

activating enzyme X or by increasing ATP breakdown. Either action would be in accord with the irritant nature of cocarcinogens, but the latter seems the more likely, and is also a likely explanation for the cocarcinogenic aspect of irritation in general (131, 139, 181). The action of the two types of compounds is shown in Figure 3, in their proposed relation to the various aspects of normal metabolism.

8. In the cases in which the virus can be isolated, the mechanism of action is explainable on the same basis as outlined above (points 2 to 6).

9. Growth is assumed to be correlated with a lowering of the effective concentration of enzyme X, that is to say, a change in the normal balance between ATP breakdown and resynthesis, as shown in Figure 3, and discussed on page 226.

Accordingly, the reason for the unrestrained growth of cancer tissue would be that the displacement of the synthesis of enzyme X by the synthesis of the "cancer virus" would result in a prevention of the normal mechanism for stopping growth, which is considered to be the removal of essential building blocks by the action of the respiratory enzymes. In the opinion of Berrill (23*), the factor for malignancy must so affect cellular relationships that cells are constantly endeavoring to attain a condition of·tissue equilibrium which cannot be reached. We have attempted to specify the nature of this equilibrium in terms of the balance between ATP and its breakdown products.

The nine points listed above embody a working hypothesis for studying the cancer problem in terms of enzymes; a number of the points have already been checked by experimental data in the case of the succinoxidase system (163). Attempts to identify enzyme X may be facilitated by consideration of its hypothetical properties. Since tumors in general possess a glycolytic rate far in excess of their capacity for oxidation, it might be expected that enzyme X is not a glycolytic enzyme but is likely to be a respiratory enzyme. Enzyme X should be inhibited by carcinogenic chemicals or their metabolic products, and it should be deficient in cancer and embryonic tissue. It was pointed out (163) that succinic dehydrogenase meets these requirements, but this enzyme is the only one to which these criteria have been applied, and Schneider and Potter (189) have emphasized that the pyruvic oxidizing system might be even more critical than the succinic system in cancer tissue.

The fact that the Rous tumor virus molecule is a ribonucleoprotein (47), together with the fact that a number of other virus particles have been shown also to be ribonucleoproteins, including the equine encephalomyelitis virus (194) and all of the plant viruses (143*), led us to raise the question whether enzyme X might also be a ribonucleoprotein. All viruses which have been successfully purified have proved to be nucleoproteins (50*, 143*), although not all are ribonucleoproteins, since the vaccinia

virus (96, 198) and the Shope papilloma virus (17) contain desoxyribonu-
cleic acid, which in animal and plant cells is exclusively a nuclear constitu-
ent (50*, 143*). However Claude has shown (49*, 50*) that the particulate
components of both normal and tumor (48) *cytoplasm* are phospholipide-
ribonucleoprotein complexes, having dimensions and "molecular weights"
similar to virus particles, and constituting at least 10 to 15% of the dry
weight of the cells (50*). This fact gave further impetus to the idea that
enzyme X and the "cancer virus" might both be ribonucleoproteins.
Since no respiratory enzymes had ever been suggested to be ribonucleo-
proteins, and since the components of respiratory enzymes which had been
isolated might well be smaller fragments of the ribonucleoprotein complex
occurring *in vivo* (for which the "molecular weight" may be as high as
139,000,000 (203), we decided to determine whether the respiratory en-
zymes could be inhibited by the action of crystalline ribonuclease, which
was obtained from Dr. M. Kunitz. It was found that only the enzymes
which reacted with cytochrome c, including the coenzyme I–cytochrome c
reductase, succinic dehydrogenase, and cytochrome oxidase, were inhibited
by ribonuclease (167). Thus, these enzymes may be ribonucleoproteins,
since Loring (126) has shown that ribonuclease combines with the tobacco
mosaic virus, which is a ribonucleoprotein.

Much further work remains to be done before the point can be estab-
lished. The particulate components of cytoplasm will have to be sepa-
rated by nonautolytic methods and their enzymatic make-up will have to be
thoroughly studied. So far, all pure enzymes have been water-soluble, but
it is apparent that they do not all occur as such *in vivo*, since protoplasm is
not a simple aqueous solution, and since many respiratory enzymes have
been proved to be insoluble (108, 123, 206). Cytochrome oxidase, which
was recently obtained in soluble form, first had to be separated from the
particulate granules with which it is associated *in vivo* (88). Stern (202*),
Kabat (102), and Szent-Györgyi (209*) previously emphasized the con-
cept of enzyme moieties associated into a macromolecular complex. It
appears quite likely that the particulate components of cytoplasm are
ribonucleoprotein complexes in which a number of cooperating enzyme
moieties are associated into what may be a primary unit of living material.
It is this primary unit which may correspond to enzyme X, and which
may be altered to form a "cancer virus."

The ribonucleoprotein units in the cytoplasm may have their counter-
parts in the desoxyribonucleoproteins of the chromosomes in the nuclei,
whose function may be solely the transmission of the enzymatic pattern,
while the ribonucleoproteins function as enzymes. It is now known that

succinic dehydrogenase, for example, is not found in nuclei but is restricted to the cytoplasm (59, 191, 231). It is believed by those who have studied the problem that the ribonucleotides and desoxyribonucleotides are metabolically related and that they may be interconvertible (143*). It is possible that differentiation represents a shift toward the ribonucleotides, while dedifferentiation or hyperplasia represents a shift toward the desoxyribonucleotides. Mitchell (144), using the Caspersson technique of monochromatic ultraviolet photomicrography (35), reported that therapeutic doses of x-rays caused an accumulation of the cytoplasmic nucleotides, and suggested that the conversion to the nuclear nucleotides had been blocked. Using the ultraviolet absorption technique, Caspersson and coworkers (36, 37, 38), as well as Brachet (30), have noted high concentrations of ribonucleic acid in the cytoplasm of growing tissues, including tumor cells (36). The 2600 Å. band was strongest in the tissues showing maximum growth. Similar correlations seem to hold for the nuclear nucleic acids, since Masayama and Yokoyama (134) reported 2115 mg. of desoxyribonucleic acid per 100 g. of tissue in hepatomas as compared with a normal range of 700 to 900. Precancerous livers contained about 1500 mg. of desoxyribonucleic acid per 100 g. of tissue. Thus, both types of nucleic acids appear to be increased in tumor tissue, and it is possible that ribonucleic acid synthesis in the cytoplasm precedes the formation of desoxyribonucleic acid in the nucleus, as has been suggested (143*).

Claude has pointed out (50*) that "so far, any organic structure which has been found to possess directly the property of self-duplication has also been shown to contain nucleic acid of the one or the other type. . . . The findings that the small particles or microsomes and the secretory granules contain ribonucleic acid suggest that these cytoplasmic constituents, like the other nucleic acid-containing structures, may be endowed with the property of self-duplication." This idea is of interest in its relation to the assumed autosynthesis of enzyme X and the "cancer virus" (point 4 of the cancer theory above). It should be emphasized that none of the self-duplicating structures may be expected to synthesize themselves in the absence of an energy source, and presumably carry out the synthesis by means of building blocks which have been phosphorylated in the course of metabolism or by interaction with ATP(see Fig. 1).

The testing of the concepts outlined in this section requires the combination of two highly technical procedures: (a) the biological and histological identification of the particles, and their separation from the other components of the cell without the loss of catalytic potency; and (b) the qualitative and quantitative measurement of the nucleic acid content and of the

specific enzymatic activities of the various types of particles.   Fortunately Claude (49*, 50*, 51) has already achieved a great deal in the separation and histological identification of the particles, although it remains to be seen whether even the precautions which he takes (low temperature, separation by centrifugal means) are sufficient to maintain the catalytic potency of some of the enzymes involved.   For many enzymes, dialysis against distilled water will cause inactivation of the protein, and the addition of a sulfhydryl compound and a continuing energy source may be needed.   The second point requires the development of specific methods of enzyme assay.   These methods must also be developed in order to assay normal and tumor tissues for various enzymatic components, and to have systems for the determination of the toxicity of carcinogenic chemicals and their breakdown products.   The development of assay methods for specific respiratory enzymes and their application to the above problems have been the chief endeavors of the author, and will be the subject of the remainder of this discussion.

III.   Methods of Assay

1.   Use of Homogenates;   Measurements of Catalytic Action

With the realization that the oxygen uptake of a tissue slice is the resultant of a large number of competing and cooperating enzyme systems (shown in Fig. 1), there has developed a need for greater knowledge of the individual systems.   It is an interesting historical fact that Warburg, whose classic paper, "Über den Stoffwechsel der Carcinomzelle" (226), established the principles of slice technique in 1924, resolutely turned away from this procedure when, in 1930, he decided that a more intimate knowledge of the specific biocatalysts was needed.   Since that date Warburg has published no papers involving the slice technique, but has carried out a series of isolations and characterizations of the specific components of respiratory enzyme systems, the first of which was the characterization of the triphosphopyridine nucleotide or coenzyme II, in 1934 (225).   As a result of the efforts of Warburg, Meyerhof, von Euler, Cori and others, we now know of many specific biocatalysts.   Furthermore, enough is known of metabolic pathways to enable us to see which specific catalysts may be expected to be isolated.   It is apparent that defects at one point or another in this mosaic of enzymatic reactions may result in pathological changes; and since the slice technique has shown that the aerobic mechanisms in cancer tissue are not adequate for the rate of glucose breakdown which

characterizes cancer tissue (226), the question which now confronts us is the finding of just which respiratory enzymes are defective in cancer tissue.

In 1936, Potter and Elvehjem (170) published a method for the disruption of animal tissues, using a device, called a homogenizer, which was constructed completely from glass. The homogenizer consists of a pestle ground into a test tube so that the fit is quite close, the combination resembling a piston in a cylinder. The end of the pestle has cutting teeth which are ground parallel to the bottom of the test tube; and the pestle is motor driven at about 1000 r.p.m., preferably by a friction drive. The tissue is ground in the presence of the desired quantity of distilled water or buffer. The resulting preparation is conveniently referred to as an "homogenate" (161), a word which has its analogy in the words filtrate, eluate, distillate, etc. The preparation is not truly homogeneous, but with proper precautions about 95% of the cells can be disrupted.

A fairly accurate estimate of the degree of dispersion of normal cells can be obtained by determining the rate of succinate oxidation with and without added cytochrome c (190). This is because the amount of cytochrome c required to saturate the succin-oxidase system is known to be about 10^{-5} moles per liter (161) and the cytochrome c content of intact cells (168) in the case of liver, muscle, and kidney are at this level or above. The dilution of the tissue is such that the cytochrome c concentration, when none is added, is low enough virtually to stop the oxidation of succinate by the disrupted cells, as can be calculated with the data given (161, 168, 190) and as has been found by experience (161, 168). Since succinate oxidation in the intact cells is the same as in homogenized cells fortified with cytochrome c, one can obtain a measure of the degree of homogenization by dividing the succinate Q_{O_2} in the absence of added cytochrome c by the succinate Q_{O_2} in the presence of added cytochrome c. In routine assays, this figure has been between 80 and 90% in all tissues except heart and skeletal muscle, in which the average ratio has been 57 and 66%, respectively (190). All these preparations were homogenized in ice-cold distilled water, which gives a higher percentage of broken cells than can be obtained with isotonic media. Elliott and Libet (73) utilized this fact in dispersing brain tissue with the homogenizer in such a way as to minimize cellular disruption, but there is no definite measure of the percentage of whole cells remaining in their preparations. Our homogenates have been shown to contain an heterogeneous collection of particles (161), which are analogous to the ribonucleoprotein complexes described by Claude (49*), and in addition a high percentage of intact nuclei. Pre-liminary studies have shown that the nuclei do not possess succinoxidase activity (191), in confirmation of earlier work (59, 231); but it is not yet possible to state quantitatively the degree of nuclear disruption, which might be measured on the basis of desoxyribo-nucleic acid determinations on the supernate from a centrifuged homogenate. Homo-genization of tissues in distilled water does not denature the proteins in the cold and in the short period of exposure prior to addition to a medium containing substrate and buffer (190). On standing there is loss of enzyme activity.

It was shown (170) that the oxygen uptake by an homogenate is affected to a marked extent by the degree of dilution. The lowering in Q_{O_2} is not proportional to the dilution but is much greater. This effect has been defined as "the dilution effect" (161, 170), and has been shown to be due to the dilution of essential cofactors in systems which require several components. In addition to the mere fact of dilution is the fact that the loss of continuing energy sources and the mixing of activators with their corresponding enzymes (especially the release of calcium ions to ATP-ase) result in a rapid breakdown

of ATP and the coenzymes of similar nature. Coenzyme I is rapidly destroyed (91), with a consequent cessation of glycolysis as well as respiration. Adenosine deaminase is probably also active in these preparations, and its action converts adenosine to NH_3 and inosine, which is much less effective as a phosphate acceptor (113).

Potter and Elvehjem (170) were aware that these reactions were occurring in the homogenates, and suggested that the preparation could be used for the study of single systems, which could be manipulated by the addition of selected substrates and cofactors, and in which the endogenous respiration could be reduced or eliminated by means of the dilution effect. Subsequent work has demonstrated the applicability of that principle. It was stated at that time that, for obvious reasons, the new technique was not intended as a substitute for the slice technique, since the two techniques yield two different kinds of information. The slice technique reveals the over-all activity of the tissue and tells essentially nothing about the individual biocatalysts which it contains. The homogenization technique, on the other hand, permits the determination of the potential activity of individual enzyme systems and reveals essentially no information about the over-all activity of the tissue. The two techniques supplement each other; and there is no point in discussing their relative merits because they measure different quantities.

The use of tissue extracts for the study of enzymes is as old as the knowledge of the existence of enzymes; but in determining the *mechanism* of enzyme action the investigator is not required to obtain a quantitative yield of any given enzyme from the tissue. Enzyme preparations which are obtained by means of fractionations or extractions cannot be used to determine the *quantity* of any given enzyme in a tissue because the yield may vary from one tissue to another and the yield cannot be obtained unless the enzyme content of the tissue can be obtained directly. If this can be done there is no point in fractionating. Homogenates have been used as the material for determining the enzyme concentration in tissues because, by this technique, it is possible to disperse cellular components so widely that endogenous respiration is virtually stopped, and because particular phases of metabolism may be studied by adding only the substrate and cofactors which are required for any given reaction. The goal is to isolate a particular *reaction* and, by so doing, to determine quantitatively the specific enzyme which catalyzes the reaction (190). The method can be applied to small samples, and can be carried out rapidly. It remains to be seen how many enzymes can be successfully studied by this technique, but several applications will be described.

Studies with the cytochrome oxidase system (190) yielded the surprising result that the various compounds which have been used for the chemical reduction of cytochrome c (hydroquinone, ascorbate, p-phenylenediamine) do not appear to penetrate the intact cells which may be present in a homogenate, in contrast to sodium succinate, which penetrates readily. In these intact cells, the endogenous Q_{O_2} is very low and the permeability is

probably not the same as *in vivo*.   When cells are taken from their normal tissue environment or broken up and dispersed, there are undoubtedly many subtle changes in the properties of the various proteins which possess catalytic activity.   The results with homogenates, as with slices and extracts (118), must therefore be examined in the perspective of the information furnished by other techniques.

Thus far, three methods have been worked out using the homogenate as the test material.   We take the position that an assay method is not valid until it has been shown that: (*a*) one specific enzyme is controlling the rate of the reaction studied; (*b*) the rate measured is linear; (*c*) the rate is directly proportional to the amount of tissue used in the test, (*d*) either no fractionation has been carried out or in any fractionations that have been made the yield is known; and (*e*) the method has been used on enough tissues to show its applicability.   In addition, the tests should employ only physiological components if possible.   In the cases mentioned below, these criteria have been met in the case of succinic dehydrogenase, cytochrome oxidase, and adenosinetriphosphatase, and appear feasible in the case of malic dehydrogenase and the coenzyme I–cytochrome c reductase.   The oxidation of pyruvate has not as yet been resolved into its component parts, but is included in order to show what factors may be needed.   Methods for the other enzyme systems shown in Figure 1 have not yet been carried to the point of satisfying the above requirements.

2.   Cytochrome Oxidase

Most respiratory enzymes have been shown to be linked to oxygen through the mediation of the cytochrome c–cytochrome oxidase system, which is shown at the far right in Figure 1.   Since this enzyme has to transmit electrons from all of the systems which reduce cytochrome c, one would expect its electron-transmitting capacity to exceed that of any individual cytochrome reductase.   Similarly, in the case of the coenzyme I–cytochrome c reductase, one would expect to find a greater electron-transmitting capacity than in any of the individual dehydrogenases which reduce coenzyme I.   Our experience has been that this relation holds for the normal tissues thus far examined.   This fact is technically very helpful because it makes possible the measurement of each of the enzyme components in the electron-transmitting sequence in a system in which the entire sequence is required for actual uptake of oxygen..   However, it must be demonstrated for each tissue that all enzymatic components except the one being measured are present in excess.   Since cytochrome oxidase is the enzyme which accordingly must be in excess of all the other components which are to be

measured, it is desirable to have a method for this particular enzyme alone before attempting to devise assay methods for the other enzymes whose electron load must pass through cytochrome oxidase before reaching oxygen. This enzyme is the simplest to study in the electron-transmitting series because it reacts directly with oxygen. The components, in sequence are as follows: reductant (*i. e.*, hydroquinone, ascorbic acid, *et al.*) — cytochrome c—cytochrome oxidase — oxygen.

Cytochrome oxidase can be assayed by setting up conditions so that cytochrome c is reduced at a greater rate than it can be oxidized by cytochrome oxidase. In such a reaction mixture, cytochrome oxidase is the limiting factor and the amount of the enzyme is proportional to the rate of oxygen uptake. Enough pure cytochrome c is added to saturate the oxidase; and the compound which reduces cytochrome c is added in sufficient amounts so that the oxidation of cytochrome c is the limiting rate. The reaction was used for the study of the properties of cytochrome oxidase by Keilin and Hartree (108). All attempts to assay tissues for the enzyme have been based on the same principles (7, 71, 190, 192, 193, 205). Schneider and Potter (190) concluded that ascorbic acid is superior to other compounds which have been used for the reduction of cytochrome c, and compared their results with those of previous investigations. The new method gives much higher results but the relative amounts found in the various tissues are about the same by all methods.

3. Succinic Dehydrogenase

The quantity of succinic dehydrogenase can be assayed in tissue homogenates by setting up a system in which this enzyme is the limiting factor (190). The sequence of reactants is as follows: succinate — succinic dehydrogenase — cytochrome c — cytochrome oxidase — oxygen. Thus far, all tissues have been found to contain cytochrome oxidase in wide excess (190), so this enzyme is not limiting. Succinate and cytochrome c are added as such in considerable excess, so that succinic dehydrogenase is the limiting factor. There are, however, two more components which must be added in order to obtain a valid measure of succinic dehydrogenase. The need for calcium was discovered by Axelrod, *et al.* (6), who showed (208) that the calcium effect was probably indirect. Through hastening the destruction of coenzyme I the calcium prevents the formation of oxalacetate from the fumarate which results from the oxidation of succinate (see Fig. 1). Unless this is done, enough oxalacetate is formed to inhibit succinic dehydrogenase, for which it has a very high affinity. In the presence of calcium, no fumarate is oxidized and the sole reaction is the oxidation of succinate. The second factor which must be added is aluminum ions, which also appear to act indirectly. It has been suggested that they prevent the destruction of the essential SH groups on succinic dehydrogenase (190) which are known to be extremely sensitive to sulfhydryl poisons (169).

Previous attempts to measure succinic dehydrogenase have usually involved the Thunberg technique; but the toxicity of methylene blue for the succinate system and its ineffectiveness compared with cytochrome c (161) make it unsuitable for studies which are to be correlated with other measurements using physiological systems.

4. Coenzyme I–Cytochrome c Reductase and Malic Dehydrogenase

In view of the rapidity with which coenzyme I is broken down in tissue homogenates (91), it might be anticipated that attempts to study the enzymes which react with coenzyme I would be futile. However, the finding that the coenzyme is protected by the addition of nicotinic acid amide (91, 132) makes possible a study of these enzymes in homogenates. Preliminary studies indicate that assays for the components of the malic system are feasible. This intermediate is oxidized via coenzyme I, but the product, oxalacetic acid, inhibits the oxidation of malic acid, probably because the potential of the malate system is higher than the potential of the coenzyme system. However, the transaminase system which involves glutamic and oxalacetic acids is so powerful (39*) that it can be used to remove oxalacetic and thus permit malate oxidation (207). The sequence of reactants in this system are: malate — *malic dehydrogenase* — coenzyme I — *coenzyme I-cytochrome c reductase* — cytochrome c — *cytochrome oxidase* — oxygen.

The italicized enzymes are present in the homogenate; since both the cytochrome reductase and the cytochrome oxidase are present in excess and the side reactions can be controlled with nicotinamide and glutamate, the limiting enzyme is malic dehydrogenase. Proof that the cytochrome reductase is present in excess is obtained by adding a Meyerhof muscle extract, which contains no cytochrome reductase or cytochrome oxidase but is rich in dehydrogenases, including malic dehydrogenase (83). When an excess of the dehydrogenase is added, the cytochrome reductase becomes the limiting factor and can therefore be assayed in the homogenate, while at the same time proof is obtained that the system minus Meyerhof extract is a valid assay for malic dehydrogenase. It is desirable to carry out the two assays simultaneously. Other coenzyme I dehydrogenases can probably be studied by modifications of this technique.

5. Keto Acid Oxidases

The oxidation of pyruvic acid and α-ketoglutaric acid both appear to require cocarboxylase (15), but the coenzyme may be responsible for no more than the decarboxylative step (84). The oxidation of pyruvate in normal animal tissues appears to involve condensation with the oxalacetic acid, as shown in Figure 1. This was demonstrated in 1940 by Krebs and Eggleston (116), whose data show clearly that the added C_4 acid does not act catalytically, but that the amount of pyruvic acid oxidized is equivalent to the fumarate added. The pathway for the complete combustion of pyruvate appears to require the condensation with oxalacetate as an obligatory step. Numerous investigators (3, 11, 41) have shown that the C_4 acids are necessary for the oxidation of pyruvate. Colowick, Kalckar, and

Cori (41) demonstrated that succinate promoted the oxidation of pyruvate in cell-free homogenates of rabbit kidney. Potter and Schneider (172) have repeated their results and have shown that all of the C_4 dicarboxylic acids (succinic, fumaric, malic, and oxalacetic) are effective, but the reconstructed system, though successful with *rabbit* kidney homogenates, does not function with cell-free *rat* kidney preparations. Colowick, in a personal communication, has confirmed this.

We have shown that guinea pig kidney behaves like rabbit kidney. The reason for the difference is not known at present, but the complexity of the system is such that any of several factors could be operative. Colowick *et al.* (41) fortified their preparation with adenylic acid, magnesium, and cocarboxylase, in addition to succinate and pyruvate. We have shown that glutathione, coenzyme I, ATP, and sodium chloride also exert marked effects on the system, although the action of the last two compounds seems to be indirect. Barrón (13*) has stated that the pyruvate-activating enzyme is one of the most sensitive of SH enzymes, and has reported stimulatory effects with glutathione (14). Sodium chloride was found by Elliott and Elliott (66) to increase the oxidation of pyruvate, malate, and similar substrates. It is apparent that considerable work remains to be done before the role of each adjuvant can be established and the system so arranged as to measure the desired enzyme, but the significant fact which is emphasized here is that this oxidation will take place in cell-free extracts. The assay can be worked out eventually.

6. Adenosinetriphosphatase

In the section on ATP utilization, it was pointed out that the protein, myosin, appears to be identical with ATP-ase. It has been possible to develop an assay for this enzyme in other tissues as well, using the homogenization technique (63).

The tissue is homogenized and studied at an extremely high dilution, that is, 0.5 to 2.0 mg. of fresh tissue in a volume of 0.65 ml. The measurement is based upon the rate of release of inorganic phosphate from ATP, in the presence of an optimum calcium concentration. There is a negligible amount of phosphate split when adenylic acid is used as a substrate instead of ATP, showing that the reaction does not proceed beyond this stage. Experiments with ADP show that this compound can substitute for ATP to some extent (171). Kalckar's studies (40, 107), however, show that there is an enzyme, myokinase, which dismutes ADP to ATP and adenylic acid, and that this enzyme could convert one-half the ADP to ATP, which could then be dephosphorylated by ATP-ase. There is the possibility that ADP can be dephosphorylated directly, although no evidence is available on this point.

7. Determinations Reported in Absolute Amounts

The enzymes reported in the preceding section cannot be assayed in absolute amounts, but only in terms of their catalytic effect, which may then be correlated with the over-all Q values for slices or whole organs.   In contrast to these enzymes, there are a number of biocatalysts which can either be measured in absolute terms or can be compared with the catalytic effects of pure standards and reported in absolute terms.   Of considerable interest to the cancer problem is the determination of cytochrome c.   The key position of this respiratory biocatalyst is evident from Figure 1 in which it is shown that the bulk of the electron load is transmitted through this carrier. The marked change in the absorption coefficients of oxidized and reduced cytochrome c at wave length 5500 Å, as well as the molecular extinction coefficients of the two forms, is known from the work of Theorell (213).   A spectrophotometric determination based on the measurement of $\Delta E550$ following enzymatic oxidation and reduction has been published by Potter and DuBois (168), while Stotz (205) has reported a measurement based on the catalytic effect in a cytochrome oxidase preparation and comparison with a standard.

Rosenthal and Drabkin (179) have recently published a microspectrophotometric method for the determination of cytochrome c which yielded higher results for the tissues reported (liver and kidney) than Potter and DuBois obtained (168).   Rosenthal and Drabkin used sodium hydrosulfite to reduce cytochrome c, the extinction of which was measured only in the reduced state.   These authors pointed out that their method eliminates the necessity of taking readings on the cytochrome in the oxidized and reduced states as well as of the preparation of a fresh enzyme for each series of analyses. We had eliminated the use of sodium hydrosulfite because of its nonspecificity, and used the enzymatic method because of the presence of interfering substances which Rosenthal and Drabkin have apparently eliminated, although the chief innovation in their method, the use of a long capillary absorption cell, makes their method even more susceptible to impurities.   The elimination of the specific oxidation and reduction of cytochrome c by enzymatic means on the basis of the labor involved in preparing the enzyme seems rather unfortunate, since the enzyme is merely a diluted kidney homogenate which can be prepared from one rat kidney in about five minutes.   The enzyme strength can vary considerably and in routine determinations never needs to be standardized.

Coenzyme I has been assayed in the von Euler and Myrbäck laboratories for many years by means of a measurement of its catalytic effect in a yeast apozymase fermentation system (148*).   Axelrod and Elvehjem (5) have used the apozymase technique for the assay of coenzyme I in animal tissues, which were frozen with solid carbon dioxide and powdered in the frozen state before extracting at 100° C.   A method based on the catalytic effect of coenzyme I on glycolysis in a Meyerhof muscle extract has been described by Jandorf, et al. (99); but this method has not been used for tissue assays.

The important position of coenzyme I in both glycolysis and respiration is exhibited in Figure 1, which shows that coenzyme II also occupies a

strategic position in the electron transmitting system. No assays for co-
enzyme II have been published as such, although assays have been pub-
lished (78) based on the zwischenferment of Warburg, Christian, and
Griese (225). A method which includes both coenzyme I and coenzyme II
is available in the form of the V-factor bacterial growth assay method of
Kohn (114); a more specific method would be desirable, since this method
includes nicotinamide nucleoside (188).

All the biocatalysts which oxidize the coenzymes have thus far been
shown to be flavoproteins. Since the high aerobic glycolysis of tumor tis-
sue indicates an inability to oxidize coenzyme I as fast as it is reduced, the
primary defect could be due to a flavoprotein deficiency as readily as to any
other oxidative enzyme. Flavin may occur both as riboflavin phosphate
(90) and as flavin-adenine-dinucleotide (223) in tissues. The former has
been determined both chemically (97) and biologically on the basis of bac-
terial growth assays (199). The dinucleotide has been determined in tis-
sues (155) on the basis of its catalytic activity in the d-amino acid oxidase
system (223).

In addition to the above cofactors, there are numerous B vitamins, some
of which have been shown to enter into the structures of cofactors and some
of which have functions that are still obscure. These include pantothenic
acid, biotin, nicotinic acid, pyridoxine, inositol, thiamine, and folic acid.
Assays for these compounds have been based on the growth response of
various bacteria on special culture media which are designed to furnish all
other growth factors. The methods, literature, and numerous applica-
tions are contained in several publications from the Texas group (158, 210,
211, 214, 215).

IV. Assay Results

1. Components of the Succinoxidase System

The succinoxidase system, according to present knowledge, consists of
only three components: succinic dehydrogenase, cytochrome c, and cyto-
chrome oxidase. Studies on the total system have indicated that cancer
tissues contain less succinoxidase than do normal tissues (53, 69); and
subsequent studies have been made on the individual components. So
much of the research on cancer has consisted of attempts to find qualita-
tive differences between cancer tissues and normal tissues that it is nearly
impossible to compile tables which compare the results of various investiga-
tors. In most cases, only one or two types of tumor were investigated and
the number of samples and the range of values are not given. In addition

the methods have varied so much that comparisons are meaningless. This is especially true for the components of the succinoxidase system, in many cases because the finding was incidental to a larger investigation or to the

TABLE II

COMPONENTS OF THE SUCCINOXIDASE SYSTEM IN NORMAL AND TUMOR TISSUES

Tissue	Succinic dehydro-genase (189)		Cytochrome c			Cytochrome oxidase (189)	
	No. of samples	Q_{O_2}	No. of samples	γ/g. wet wt.	Ref. No.	No. of samples	Q_{O_2}
Normal rat:							
Heart	6	219	10	371	61	6	974
	..	...	10	530	205	..	...
Kidney	6	195	10	247	61	6	549
	..	...	10	330	205	..	...
Liver	7	87.7	10	90	61	7	392
	..	...	10	68	205	..	...
Brain	6	48.7	10	50	61	6	420
	..	...	10	75	205	..	...
Skeletal muscle	6	35.5	10	97	61	6	180
	..	...	10	160	205	..	...
	..	...	..	90	77*	..	...
Spleen	6	23.3	10	43	61	6	195
	..	...	10	48	205	..	...
Lung	6	17.9	10	21	61	6	92.3
	..	...	10	29	205	..	...
Rat liver tumor	4	26.3	11	20	61	4	134
Hepatoma 31, rat	7	21.7	..	...	...	7	136
Transplant, rat hepatoma	4	18.1	..	...	...	4	94
Walker 256 rat carcinoma	6	9.4	11	9	61	6	61.5
Flexner-Jobling rat carci-noma	7	15.5	14	12	61	7	91.3
Yale No. 1 mouse tumor	6	20.2	12	16	61	6	106
Jensen rat sarcoma	7	17.8	7	12	61	7	129
	..	...	?	3	77*	..	...
Mouse ear tumor	10	19.1	8	11	61	10	64
Rous chicken sarcoma	10	11.1	12	12	61	10	44.4
Mouse tumor, 2,2-azo-naphthalene	..	...	7	15	61	..	...
Mouse mammary tumor	10	27.7	6	14	61	10	87.8
Tumor R-256	..	...	3	3	205	..	...
Tumor R-39	..	...	3	5	205	..	...
Spontaneous	..	...	1	2	205	..	...
Human liver carcinoma	..	...	1	3	101	..	...

development of a method. The assays for the components of the succin-
oxidase system were developed and applied to a number of experimental
tumors in this laboratory (61, 189) as shown in Table II, which includes
some additional values for cytochrome c as reported from other laboratories.

Von Euler (77*) has placed considerable emphasis on the cytochrome c deficiency in
tumors, although the deficiency appears to be less marked than his figures for Jensen
sarcoma would indicate. DuBois and Potter obtained consistently higher values for
tumors than did the other investigators, and called attention to the errors due to loss
in tissues which are originally low in cytochrome c.

The data show that there is a striking uniformity in the tumor values, whereas the
cytochrome c and other data in Table II show that normal tissues vary from tissue to
tissue by more than tenfold. This suggests that tumors may represent a uniform tissue
of type regardless of origin. The uniformity of tumors has been stressed on the basis
of assays for the B vitamins (212), but has been questioned on the basis of certain enzyme
studies, particularly the acid and alkaline phosphatases (85).

Rosenthal and Drabkin (179a) found that the cytochrome c content of several rat
tumors approximated the values reported by DuBois and Potter. They also obtained
approximately the same values for the normal rat tissues analyzed by the previous
workers, although the values for liver and lung differed markedly from the earlier data.
Without attempting to explain the fact that the rat hepatomas contained about one-fifth
as much cytochrome c as normal rat liver, they report that, in the case of rat mammary
gland, the cytochrome c concentration was in the tumor range and not significantly
different from a specimen of spontaneous mammary rat tumor. Similarly it was re-
ported that samples of normal colon mucosa of man were not significantly richer in
cytochrome c than neoplasms derived from this tissue. It is interesting to note that
normal rat mammary tissue studied was obtained from rats in the fourteenth day of
pregnancy, and was in a state of hyperplasia; they were unable to obtain the tissue from
nonpregnant rats. We were aware that tumor tissue is not unique with respect to its
cytochrome c content, inasmuch as we published a table showing that embryonic tissue
contains even less cytochrome c than any of the tumor samples. It appears that the
cytochrome c content of tumors is not much different from that of other undifferentiated
growing tissues. This observation would be in harmony with the mechanism of normal
and of malignant growth outlined in an earlier section. The mechanisms described
would also provide a basis for the statement by Rosenthal and Drabkin that, with
respect to species as well as tissue comparison, there was a correlation between low
cytochrome and spontaneous incidence of tumors.

The remaining components of the succinoxidase system rather closely
parallel the values for cytochrome c, which suggests that the three bio-
catalysts may be part of a complex formed more or less as a unit. Suc-
cinic dehydrogenase (71) and cytochrome oxidase (71, 193, 205) have been
studied by other investigators and the results, though quantitatively quite
different from the values in Table II (see 190), show the same relative dif-
ferences between normal and tumor tissues. In continuation of the earlier
studies on animal neoplasms, the Yale group (180a) have measured the

over-all succinoxidase system in a large number of human neoplasms which were compared with homologous normal human tissue. The findings completely confirmed their earlier studies on animals, which are in line with the results on the individual components of the succinoxidase system, as shown in Table II. If the results of Rosenthal and Drabkin (179a) can be accepted, it may be inferred that, in the case of human neoplasms, the succinic dehydrogenase is a more critical factor than cytochrome c. Roskelley, et al. (180a) also included an excellent study of succinoxidase in rat liver during the production of hepatomas by feeding p-dimethylaminoazobenzene. Initially the increase in Q_{O_2} due to succinate was larger than in the case of control rats, but the succinate effect subsequently declined until finally no response was obtained. This point coincided with the appearance of frank cancer. Again it may be emphasized that these findings find a ready interpretation in terms of the cancer theory described earlier.

In general, the results on the tumor tissues show that the components of the succinoxidase system are not missing but are considerably lower in amount than the active normal tissues. However, certain normal tissues are just as low as are the tumor tissues   Probably the most valid comparison in Table II may be made between normal liver and liver tumor. In this case, it is seen that the tumor tissue contains only 20 to 30% as much of any component of the succinoxidase system as is found in normal liver. On the other hand, the normal tissues which are high in succinoxidase in the adult, when studied in the embryonic or new-born rat, appear to contain amounts comparable to the amounts in tumor tissue (172). It thus appears that, with respect to the succinoxidase system, as in the case of anaerobic and aerobic glycolysis, tumor tissue resembles embryonic tissue. The amount of succinoxidase does not appear to be critical in either normal or tumor tissue because the oxygen uptake of slices on glucose is always lower than would be possible on the basis of the succinoxidase content. This suggests that one of the limiting reactions in carbohydrate oxidation lies prior to the succinate step (178). Since lactate–pyruvate accumulates in tumor tissue, it may be suggested that the deficiency may be sought in the stages between pyruvate and succinate (189). The oxidation of pyruvate and α-ketoglutarate both occur in this pathway, and both reactions are oxidative decarboxylations. No assay methods are as yet available for the study of these specific reactions.

2. ATP-ase

The results with the succinoxidase system (178, 189) suggests that, in normal tissue, the rate of glycolysis may be a limiting factor in oxygen

uptake, whereas the marked accumulation of lactate–pyruvate in tumor tissue shows that the rate of glycolysis is not limiting but that some later reaction is deficient. It has been shown how the breakdown of ATP influences glycolysis (page 221, also reference 61). The determination of ATP-ase concentration in tumor tissues therefore is of interest. Data on liver and liver tumors are presented in Table III, although a complete survey of various tumor types is not available. It is evident that, in this case at least, the ATP-ase content of the tumor does not resemble that of the embryonic tissue, but that it compares favorably with the adult tissue. What is really needed is a determination of the state of activity of ATP-ase in the two tissues; but on the basis of the physiology of the two tissues one

TABLE III

ADENOSINETRIPHOSPHATASE CONTENT OF LIVER AND LIVER TUMORS[a]

Rat tissue	No. of samples	ATP-ase units per mg. fresh tissue	
		Maximum variation	Average
Normal adult liver	8	±3.5	12.3
B-Y induced liver tumor	8	±3.0	11.3
Late embryonic liver:			
18–21 days	7	±1.0	3.0
Young rat liver			
1–5 days	7	±3.0	6.8
5–10 days	5	±2.0	8.7
10–25 days	7	±2.7	9.5

[a] Method from (63). Data from (63, 171).

is tempted to suggest that the ATP-ase in the hepatoma is fully activated while that in liver is activated in proportion to the demands of the rest of the body. However, even if the activation is no greater than in normal liver, we have here an example of an enzyme which is not low in tumor tissue when compared to homologous normal tissue and which is present in higher amounts in tumor tissue than in homologous embryonic tissue. There is one other enzyme known for which the situation appears to be similar. Greenstein, et al. (85) have reported that the splitting of glycogen occurred just as rapidly in liver tumor as in normal liver. Thus, the two enzymes which are not low in tumor tissue are both concerned with the glycolytic process, while the enzymes which appear to be defective are in the aerobic mechanism.

3. Cofactors

One of the most interesting cofactors is coenzyme I (Co I), otherwise known as cozymase, codehydrogenase I, or diphosphopyridine nucleotide. As can be seen in Figure 1, this coenzyme occurs both in the glycolytic mechanism and in the aerobic mechanism. In the embryonic and new-born rat tissues, the concentration of Co I is low. During the first few days of life, the amount quickly increases to the adult level (22). These assays in-

TABLE IV

COENZYME CONTENT OF NORMAL AND TUMOR TISSUES IN γ PER GRAM WET WEIGHT

Tissue	Coenzyme I			Coenzyme II			Coenzymes I and II*		
	No. of samples	Result	Ref. No.	No. of samples	Result	Ref. No.	No. of samples	Result	Ref. No.
Rat liver	8	1390	110	?	30	78	15	542	22
	?	215	78	..	..	..	..	...	..
Rat kidney	?	160	78	?	40	78	14	510	22
Rat muscle:									
Adult	?	160	78	⸂	80	78	10	522	22
Young	?	200	...	?	80	78	..	..	..
Rat spleen	..	..	...	..	..	..	7	568	22
Rat heart	?	150	78	?	40	78	..	...	..
Jensen sarcoma	?	160	78	?	80	78	..	..	..
Walker 256	..	..	...	..	..	..	12	71	22
B-Y tumor (dietary)	8	150	110	..	..	..	..	...	..

* V-factor assay.

cluded both Co I and Co II. Evidently glycolysis can proceed in the presence of a much lower concentration of Co I than is required for the maintenance of glycolysis *and* respiration. No real survey of coenzymes I and II in tumor tissues in general has yet been made, but available data (Table IV) indicate that the coenzymes may be lower in the tumors than in homologous control tissue. Considerably more work is needed to establish the point. The breakdown of these coenzymes is very rapid and tumors probably should be frozen *in situ*, since the destruction in tumors may exceed that in other tissues (28, 29). Assays of tumor tissues for the enzymes which split the coenzymes would probably be of considerable interest, because normal tissues show considerable variation (91). Coenzymes I and II need to be determined by specific methods, but their probable interconvertibility *in vivo* should not be forgotten (1).

TABLE V

RIBOFLAVIN CONTENT OF NORMAL AND TUMOR TISSUES
IN γ PER GRAM WET WEIGHT

Normal tissues	No. of samples	Results	Ref. no.	Tumor tissues	No. of samples	Results	Ref. no.
Rat liver:				Rat hepatoma:			
Stock diet	?	29.3	111	Stock diet plus "butter yellow"	?	5	111
	8	29.4	177		6	4	177
Purina diet	2	28	157	Purina diet	2	5.7	157
Rice–carrot diet	2	13	157	Rice–carrot diet	1	3.4	157
	?	13.2	133		?	2.6	133
	?	17.2	111		..	..	...
Fetal	2	5.5	177	Rat Walker 256	3	3.2	157
Regenerated	6	24.7	177	Rat hepatoma 31	18	4.8	177
Liver:				Rat Jensen sarcoma	6	3.6	177
C$_{57}$ mouse	3	41	157	C$_{57}$ mouse sarcoma (m e t h y l c h o l-anthrene)	3	6	157
dba mouse	3	31	157	dba mouse adeno-carcinoma	3	3.2	157
C$_3$H mouse	3	25	157	C$_3$H mouse adeno-carcinoma	5	2.1	157
Heart:				Mouse lymphosar-coma	4	3.7	177
Wistar rat	2	12	157	Mouse intestinal carcinoma	3	3.9	177
C$_{57}$ mouse	3	36	157				
dba mouse	3	20	157	Mouse salivary gland tumor	4	3.5	177
C$_3$H mouse	3	36	157	Mouse ear tumor, UV irradiation	5	4.4	177
Brain:				Mouse malignant melanoma S 91	14	5.6	177
Wistar rat	2	3.1	157				
C$_{57}$ mouse	3	4.3	157	Mouse sarcoma 37	4	3.7	177
dba mouse	3	2.4	157	Mouse Crocker sar-coma 180	4	4.1	177
C$_3$H mouse	3	3.4	157	Mouse brain tumor	6	2.4	177
Lung:				Mouse spontane-ous mammary adenocarcinoma	5	5.5	177
Wistar rat	2	4.4	157				

TABLE V (*Continued*)

Normal tissues	No. of samples	Results	Ref. no.	Tumor tissues	No. of samples	Results	Ref. no.
Spleen: Wistar rat	2	3.3	157	Mouse adenocar- cinoma of glandu- lar stomach	6	4.3	177
Kidney: Wistar rat	2	28	157	Mouse squamous cell carcinoma of fore-stomach	6	3.3	177

Skeletal
  muscle:
  Wistar rat
	2	1.9	157	Mouse hepatoma
				(azotoluene)
					29	8.4	177

				Mouse spontaneous hepatoma	10	15.3	177
				Mouse hepatoma (CCl₄)	7	15.2	177

Flavin–adenine dinucleotide determinations do not appear to have been made on tumor tissues, although Warburg and Christian report that it is present in Jensen sarcoma (223). A considerable number of assays for flavin have been carried out; these probably represent the sum of flavin plus flavin–adenine dinucleotide. The data are presented in Table V. It is fairly clear that cancer tissues tend to be low in flavin, and to an extent which is comparable to the components of the succinoxidase system. It is also significant that flavin rises from the embryonic level to the adult level in a few days after birth, during the same period that the aerobic enzymes are increasing. There are many enzymes which are flavoproteins; and the significance of the flavin determinations will not be appreciated until the specific enzymes have been studied. So far, the enzymes which oxidize the coenzymes have been shown to be flavoproteins; the coenzyme I–cytochrome c reductase may be in this group. This enzyme in particular should be determined in cancer tissue because it is one of the most likely bottlenecks in the electron-transmitting system in cancer tissue. Von Euler, *et al.* (78) have reported that, in two samples of Jensen rat sarcoma, the ratio of reduced to oxidized Co I was 6.2 and 8.5, while the ratio in muscle was 0.56 and 1.0, which indicates a lack of cytochrome reductase in the sarcoma. This highly significant observation needs wider application and confirmation.

Assays of tumor tissues for thiamine are of interest because, when this

vitamin is phosphorylated, it becomes the coenzyme for the α-keto acid oxidases. Assays (215) show that the thiamine content of liver tumors is similar to that of other tumors. The tumors contain less than liver tissue but about the same amount of thiamine as muscle tissue.

West and Woglom (227) have studied the biotin content of tumors and embryonic tissues. They found that certain tissues showed an increase in biotin content in the transition from embryonic to adult tissues, while other tissues exhibited a decrease. In general, tumors seemed to resemble the embryonic form of the homologous tissue. The biotin content of tumors has also been studied by Pollack, *et al.* (158), as well as the content of the other B vitamins in tumor tissues (210, 211, 214, 215).

V. Inhibition of Respiratory Enzymes by Carcinogenic Chemicals

A corollary of the concept that cancer tissue is deficient in some essential enzyme component is that, in normal tissue, the particular enzyme should be damaged by carcinogenic agents. Earlier studies with the carcinogenic hydrocarbons yielded essentially negative results due to the insolubility of the hydrocarbons in aqueous systems. It was only when the hydrocarbons were partially oxidized that toxicity resulted (27); and there was no basis for assuming that the toxic compound represented a metabolite of the hydrocarbon. The concept that a product of the carcinogenic agent might be the active factor in carcinogenesis was first successfully carried through the necessary experimental steps in a series of studies by Kensler and Rhoads and their associates for the azo dye, *p*-dimethylaminoazobenzene which is generally used for the production of dietary liver tumors.

It was shown that the urine of animals fed the carcinogen contained *p*-aminopheno *p*-phenylenediamine, and the acetylated products of these compounds (204). Althoug the *N*-dimethyl-*p*-phenylenediamine was not found, it was thought to be a possibl intermediate between the carcinogen and *p*-phenylenediamine. Since Kensler, *et a* (110) had found a lowering in the coenzyme I content of the liver tumors, they nex turned their attention to a Co I system as a logical site of action for the metabolites c the carcinogen. They were able to show that the yeast apozymase system which ha been used for the Co I assays was inhibited by the *p*-substituted metabolites but not b the acetylated products; and it was considered that the latter represented the produc of detoxification mechanisms (109). It was further shown that the apozymase syste was protected by the coenzyme. Similar results were obtained with the yeast ca boxylase system (112). They stated that, the toxicity of the intermediates parallele the carcinogenicity of the parent azo dyes, and the enzymes were protected by su hydryl compounds, suggesting that a possible mechanism of toxicity was interactio between essential SH groups of the enzymes and the metabolites of the carcinogen.

Potter (162) showed that essentially all the observations which had bee made on the yeast enzymes could be duplicated with jack bean ureas

It was suggested that the toxicity of the compounds was associated with their autoxidizability rather than with their free radical stability. Potter and DuBois (169) then took up the problem of attempting to localize the site of toxic action in terms of animal enzyme systems, since it was felt that the inhibition of the yeast system and the urease system was of significance only insofar as it emphasized sulfhydryl systems. That the toxic agents were acting on the glycolytic enzyme system seemed improbable because of the known high rate of glycolysis in liver tumors. The succinoxidase system was fairly well in hand by this time, and a thorough study was made of the toxic action of all the breakdown products of the azo dye, plus a number of closely related compounds and heterogeneous inhibitors of SH enzymes. It was found that compounds of the quinonoid structure were extremely toxic to the succinic dehydrogenase, such compounds being readily formed from p-aminophenol, N-dimethyl-p-phenylenediamine, and the other non-acetylated breakdown products of p-dimethylaminoazobenzene, by oxidation through the cytochrome system. Comparison of the data from the two systems (162, 169) shows clearly that the metabolites of the carcinogen are nontoxic until oxidized. This fact may be of great significance in carcinogenesis, since in normal tissues the toxic agent would be formed by oxidation via the cytochrome system and could then act on succinic dehydrogenase, which is so closely associated with the cytochrome system. The triose phosphate dehydrogenase, which is the most sensitive SH enzyme in the glycolytic complex, would be protected by its coenzyme (109, 169). With the reduction in the succinate system, the Krebs cycle would fail, and there would be a shift toward the glycolytic type of metabolism which is characteristic of tumors. In areas where this change had taken place, the cancer type of metabolism would not be as effective for the oxidation of the dye intermediates and would thus be less susceptible to the dye. This is not to say that succinic dehydrogenase is identified as the enzyme X discussed above (page 229). More and more enzymes have been recognized to contain SH groups which are necessary for their catalytic action (14). One of the enzymes which should be studied in terms of SH poisons is the pyruvic oxidizing system, which, according to Barrón (13*), is one of the most sensitive SH enzymes. Another enzyme that should be studied in these terms is the coenzyme I–cytochrome reductase. Either of the above two enzymes could easily be the site of carcinogenic action, and should be studied thoroughly before too much emphasis is placed on succinic dehydrogenase. Since flavin has been considered as a probable constituent of all three enzymes, and since SH is also probably involved in this type of enzyme action, there is as yet no way of stating the

exact locus of action. However, the succinic dehydrogenase system serves as a model in illustrating the manner in which the shift from aerobic to glycolytic metabolism might come about, and the mechanism would be the same for both of the other enzymes mentioned. It is altogether likely that other carcinogenic chemicals act on SH enzymes (230). Reimann (174) has shown that simultaneous painting with thiols protects mice against 1,2,5,6-dibenzanthracene; and White (229) has shown that the toxic effects of orally ingested hydrocarbons are alleviated by administration o increased amounts of cysteine.

The finding that an enzyme contains sulfhydryl groups is not evidence that it is involved in the mechanism of carcinogenesis. The question is Which SH enzymes are most sensitive to the carcinogenic chemicals It appears that there are many criteria by which the hypothetical enzym X may be tested. There is good reason to believe that it may some day b identified, and the whole sequence of metabolic events which lead to cance may then unfold. The significance of these studies is not limited to th cancer problem alone, but embraces the whole field of medicine.

Bibliography

1. Adler, E., Elliot, S., and Elliot, L., *Enzymologia*, **8**, 80 (1940).
2. Adler, E., von Euler, H., Günther, G., and Plass, M., *Biochem. J.*, **33**, 102 (1939).
3. Annau, E., and Erdös, T., *Z. physiol. Chem.*, **257**, 111 (1939).
4. Atkin, L., *Thesis*, New York University, 1940.
5. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **131**, 77 (1939).
6. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *ibid.*, **140**, 931 (1941).
7. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *ibid.*, **145**, 297 (1942).
8. Bailey, K., *Biochem. J.*, **36**, 121 (1942).
9. Bailey, K., Astbury, W. T., and Rudall, K. M., *Nature*, **151**, 716 (1943).
10. Baker, Z., *Biochem. J.*, **32**, 332 (1938).
11. Banga, I., Ochoa, S., and Peters, R. A., *ibid.*, **33**, 1980 (1939).
12. Banga, I., and Szent-Györgyi, A., *Science*, **92**, 514 (1940).
*13. Barrón, E. S. G., *Advances in Enzymology*, Vol. III. Interscience, New Yor 1943, p. 149.
14. Barrón, E. S. G., and Singer, T. P., *Science*, **97**, 356 (1943).
15. Barrón, E. S. G., Lyman, C. M., Lipton, M. A., and Goldinger, J., *Proc. A Soc. Biol. Chemists*, **9**, xi (1941).
*16. Bayne-Jones, S., Harrison, R. G., Little, C. C., Northrop, J., and Murph J. B., *U. S. Pub. Health Repts.*, **53**, 2121 (1938).
17. Beard, J. W., Taylor, A. R., Sharp, D. G., and Beard, D., *Surg., Gynecol. Obst* **74**, 509 (1942).
18. Belitser, V. A., and Tsybakova, E. T., *Biokhimiya*, **4**, 516 (1939).
18a. Belitser, V. A., *Enzymologia*, **6**, 1 (1939).
19. Benoy, M. P., and Elliott, K. A. C., *Biochem. J.*, **31**, 1268 (1937).

20. Berenblum, I., *Cancer Research*, 1, 44 (1941).
21. Berenblum, I., *ibid.*, 1, 807 (1941).
22. Bernheim, F., and Felsovanyi, A., *Science*, 91, 76 (1940).
*23. Berrill, N. J., *Physiol. Revs.*, 23, 101 (1943).
24. Blum, H. F., *J. Natl. Cancer Inst.*, 3, 569 (1943).
25. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, 146, 673 (1942).
26. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *ibid.*, 149, 529 (1943).
27. Boyland, E., and Boyland, M. E., *Biochem. J.*, 28, 244 (1934).
28. Boyland, E., and Boyland, M. E., *ibid.*, 32, 321 (1938).
29. Boyland, E., Boyland, M. E., and Greville, G. D., *ibid.*, 31, 461 (1937).
30. Brachet, J., *Enzymologia*, 10, 87 (1941).
31. Breusch, F. L., *Science*, 97, 490 (1943).
*32. Burk, D., *Cold Spring Harbor Symposia Quant. Biol.*, 7, 420 (1939).
*33. Burk, D., in *A Symposium on Respiratory Enzymes*. Univ. Wisconsin Press, Madison, 1942, p. 235.
34. Burk, D., Sprince, H., Spangler, J. M., Kabat, E. A., Furth, J., and Claude, A., *J. Natl. Cancer Inst.*, 2, 201 (1941).
35. Caspersson, T., *Skand. Arch. Physiol.*, 73, Suppl. No. 8 (1936).
36. Caspersson, T., Nystrom, C., and Santesson, J., *Naturwissenschaften*, 29, 29 (1941).
37. Caspersson, T., and Schultz, J., *Nature*, 143, 602 (1939).
38. Caspersson, T., and Thorell, B., *Chromosoma*, 2, 132 (1941). Quoted from A. E. Mirsky in *Advances in Enzymology*, Vol. III, Interscience, New York, 1943.
*39. Cohen, P. P., *Federation Proc.*, 1, 273 (1942).
40. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, 148, 117 (1943).
41. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *ibid.*, 137, 343 (1941).
42. Colowick, S. P., and Sutherland, E. W., *ibid.*, 144, 423 (1942).
43. Colowick, S. P., Welch, M. S., and Cori, C. F., *ibid.*, 133, 359 (1940).
44. Colowick, S. P., Welch, M. S., and Cori, C. F., *ibid.*, 133, 641 (1940).
*45. Cori, C. F., in *A Symposium on Respiratory Enzymes*. Univ. Wisconsin Press, Madison, 1942.
46. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 135, 733 (1940).
47. Claude, A., *Science*, 90, 213 (1939).
48. Claude, A., *ibid.*, 91, 77 (1940).
*49. Claude, A., *Cold Spring Harbor Symposia Quant. Biol.*, 9, 263 (1941).
*50. Claude, A., *Science*, 97, 451 (1943).
51. Claude, A., and Potter, J. S., *J. Exptl. Med.*, 77, 345 (1943).
52. Claude, A., and Rothen, A., *ibid.*, 71, 619 (1940).
53. Craig, F. N., Bassett, A. M., and Salter, W. T., *Cancer Research*, 1, 869 (1941).
54. Delafield, F., Prudden, T. M., and Wood, F. C., *A Textbook of Pathology*. Wood, New York, 1927, Chapter IV.
55. Dickens, F., *Biochem. J.*, 32, 1626 (1938).
56. Dickens, F., *ibid.*, 32, 1645 (1938).
57. Dickens, F., and Weil-Malherbe, H., *Cancer Research*, 3, 73 (1943).
57a. Domini, G., and Rein, H., *Arch. ges. Physiol.*, 246, 608 (1943).
*58. Dorfman, A., *Physiol. Revs.*, 23, 124 (1943).
59. Dounce, A. L., *J. Biol. Chem.*, 147, 685 (1943).
*60. Drury, A. N., *Physiol. Revs.*, 16, 292 (1936).

61. DuBois, K. P., and Potter, V. R., *Cancer Research*, **2**, 290 (1942).
62. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, **148**, 451 (1943).
63. DuBois, K. P., and Potter, V. R., *ibid.*, **150**, 185 (1943).
64. Elliott, K. A. C., *Biochem. J.*, **34**, 1134 (1940).
*65. Elliott, K. A. C., in *A Symposium on Respiratory Enzymes.* Univ. Wisconsi Press, Madison, 1942, p. 229.
66. Elliott, K. A. C., and Elliott, F. H., *J. Biol. Chem.*, **127**, 457 (1939).
67. Elliott, K. A. C., and Baker, Z., *Biochem. J.*, **29**, 2396 (1935).
68. Elliott, K. A. C., and Baker, Z., *ibid.*, **29**, 2433 (1935).
69. Elliott, K. A. C., Benoy, M. P., and Baker, Z., *ibid.*, **29**, 1937 (1935).
70. Elliott, K. A. C., and Greig, M. E., *ibid.*, **31**, 1021 (1937).
71. Elliott, K. A. C., and Greig, M. E., *ibid.*, **32**, 1407 (1938).
72. Elliott, K. A. C., Greig, M. E., and Benoy, M. P., *ibid.*, **31**, 1003 (1937).
73. Elliott, K. A. C., and Libet, B., *J. Biol. Chem.*, **143**, 227 (1942).
74. Elliott, K. A. C., and Schroeder, E. F., *Biochem. J.*, **28**, 1920 (1934).
*75. Engelhardt, W. A., *Yale J. Biol. Med.*, **15**, 21 (1942).
76. Engelhardt, W. A., and Barkhash, A. P., *Biokhimiya*, **3**, 500 (1938).
*77. von Euler, H., *Deut. med. Wochschr.*, **64**, 1712 (1938).
78. von Euler, H., Schlenk, F., Heiwinkel, H., and Högberg, B., *Z. physiol. Chem* **256**, 208 (1938).
*79. Fieser, L. F., *Cause and Growth of Cancer.* Univ. Pennsylvania Press, Phil∢ delphia, 1941, pp. 1–27.
80. Flock, E. V., Ingle, D. J., and Bollman, J. L., *J. Biol. Chem.*, **129**, 99 (1939).
81. Furchgott, R. F., and Shorr, E., *ibid.*, **151**, 65 (1943).
*82. Gellhorn, E., *Autonomic Regulations.* Interscience, New York, 1943.
83. Green, D. E., Needham, D. M., and Dewan, J. G., *Biochem. J.*, **31**, 2327 (1937
84. Green, D. E., Westerfield, W. W., Vennesland, B., and Knox, W. E., *J. Bi∢ Chem.*, **140**, 683 (1941).
85. Greenstein, J. P., Edwards, J. E., Andervont, H. B., and White, J., *J. Na∢ Cancer Inst.*, **3**, 7 (1942).
*86. Greenstein, J. P., *Advances in Enzymology*, Vol. III. Interscience, New Yor 1943, p. 315.
87. Greig, M. E., Munro, M. P., and Elliott, K. A. C., *Biochem. J.*, **33**, 443 (1939)
88. Haas, E., *J. Biol. Chem.*, **148**, 481 (1943).
89. Haas, E., Harrer, C. J., and Hogness, T. R., *ibid.*, **143**, 341 (1942).
90. Haas, E., Horecker, B. L., and Hogness, T. R., *ibid.*, **136**, 747 (1940).
91. Handler, P., and Klein, J. R., *ibid.*, **143**, 49 (1942).
92. Harrison, S. T., and Mellanby, E., *Biochem. J.*, **24**, 141 (1930).
93. Hegnauer, A. H., Fisher, R. E., Cori, G. T., and Cori, C. F., *Proc. Soc. Exp Biol. Med.*, **32**, 1075 (1935).
*94. Heilbrunn, L. V., *Outline of General Physiology.* Saunders, Philadelphia, 193∑
95. Heilbrunn, L. V., Mazia, D., and Steinbach, H. B., *Anat. Record* (Suppl.), ∢ 32 (1934).
96. Hoagland, C. L., Lavin, G. I., Smadel, J. E., and Rivers, T. M., *J. Exptl. Me* **72**, 139 (1940).
97. Hodson, A. Z., and Norris, L. C., *J. Biol. Chem.*, **131**, 621 (1939).
98. Holmes, B. E., *Biochem. J.*, **31**, 1730 (1937).

99. Jandorf, B. J., Klemperer, F. W., and Hastings, A. B., *J. Biol. Chem.*, **138**, 311 (1941).

*100. Johnson, M. J., *Science*, **94**, 200 (1941).

101. Junowicz-Kocholaty, R., and Hogness, T. R., *J. Biol. Chem.*, **129**, 569 (1939).

102. Kabat, E. A., *Science*, **93**, 43 (1941).

103. Kahler, H. , and Robertson, W. v. B., *J. Natl. Cancer Inst.*, **3**, 495 (1943).

104. Kalckar, H., *Enzymologia*, **2**, 47 (1937).

105. Kalckar, H., *Biochem. J.*, **33**, 631 (1939).

*106. Kalckar, H. M., *Chem. Revs.*, **28**, 71 (1941).

107. Kalckar, H. M., *J. Biol. Chem.*, **148**, 127 (1943).

108. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London*, **B125**, 171 (1938).

109. Kensler, C. J., Dexter, S. O., and Rhoads, C. P., *Cancer Research*, **2**, 1 (1942).

110. Kensler, C. J., Suguira, K., and Rhoads, C. P., *Science*, **91**, 623 (1940).

111. Kensler, C. J., Suguira, K., Young, N. F., Halter, C. R., and Rhoads, C. P., *ibid.*, **93**, 308 (1941).

112. Kensler, C. J., Young, N. F., and Rhoads, C. P., *J. Biol. Chem.*, **143**, 465 (1942).

113. Kleinzeller, A., *Biochem. J.*, **36**, 729 (1942).

114. Kohn, H. I., *ibid.*, **32**, 2075 (1938).

*114a. Korr, I. M., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 74 (1939).

*115. Krebs, H. A., *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, p. 191.

116. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 442 (1940).

117. Lardy, H. A., *personal communication*.

118. Laser, H., *Biochem. J.*, **36**, 319 (1942).

119. Lavik, P. S., Moore, P. R., Rusch, H. P., and Baumann, C. A., *Cancer Research*, **2**, 189 (1942).

120. Lipmann, F., *Nature*, **138**, 588 (1936).

*121. Lipmann, F., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 248 (1939).

*122. Lipmann, F., *Advances in Enzymology*, Vol. I. Interscience, New York, 1941, p. 99.

122a. Lipmann, F., in *A Symposium on Respiratory Enzymes*. Univ. Wisconsin Press, Madison, 1942.

123. Lockhart, E. E., and Potter, V. R., *J. Biol. Chem.*, **137**, 1 (1941).

124. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).

125. Loofbourow, J. R., *Biochem. J.*, **36**, 737 (1942).

126. Loring, H. S., *J. Gen. Physiol.*, **25**, 497 (1942).

127. Lundsgaard, E., *Biochem. Z.*, **217**, 162 (1930).

128. Lundsgaard, E., *ibid.*, **233**, 322 (1931).

129. Lundsgaard, E., *ibid.*, **269**, 308 (1934).

130. Lyman, C. M., and Barrón, E. S. G., *J. Biol. Chem.*, **132**, 293 (1940).

131. MacKenzie, I., and Rous, P., *J. Exptl. Med.*, **73**, 391 (1941).

132. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).

133. Masayama, T., and Yokoyama, T., *Gann.*, **33**, 214 (1939).

134. Masayama, T., and Yokoyama, T., *ibid.*, **34**, 174 (1940).

135. Matsuzaki, H., *J. Biochem. Japan*, **17**, 457 (1933).

136. Mazia, D., *J. Cellular Comp. Physiol.*, **10**, 291 (1937).

137. Mendel, B., *Klin. Wochschr.*, **8**, 169 (1929).

138. Mendel, B., Bauch, M., and Strelitz, F., *ibid.*, **10**, 118 (1931).

139. Menkin, V., *Cancer Research*, **1**, 548 (1941).
140. Meyerhof, O., *Naturwissenschaften*, **23**, 850 (1935).
*141. Meyerhof, O., in *A Symposium on Respiratory Enzymes*. Univ. Wiscon Press, Madison, 1942.
142. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
*143. Mirsky, A. E., *Advances in Enzymology*, Vol. III. Interscience, New York, 19 p. 1.
144. Mitchell, J. S., *Brit. J. Exptl. Path.*, **23**, 285, 296, 309 (1942).
145. Munoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, **147**, 355 (1943).
*146. Murphy, J. B., *J. Am. Med. Assoc.*, **120**, 107 (1942).
147. Murphy, J. B., and Sturm, E., *Cancer Research,* **1**, 609 (1941).
*148. Myrbäck, K., *Ergeb. Enzymforsch.*, **2**, 139 (1933).
149. Needham, D. M., *Biochem. J.*, **36**, 113 (1942).
150. Needham, J., Kleinzeller. A., Miall, M., Dainty, M., Needham, D. M.. Lawrence, A. S. C., *Nature*, **150**, 46 (1942).
151. Negelein, E., and Brömel, H., *Biochem. Z.*, **303**, 132 (1939).
152. Ochoa, S., *J. Biol. Chem.*, **141**, 245 (1941).
153. Ochoa, S., *ibid.*, **151**, 493 (1943).
154. Ochoa, S., *ibid.*, **149**, 577 (1943).
155. Ochoa, S., and Rossiter, R. J., *Biochem. J.*, **33**, 2008 (1939).
156. Orr, J. W., and Stickland, L. H., *ibid.*, **35**, 479 (1941).
157. Pollack, M. A., Taylor, A., Taylor, J., and Williams, R. J., *Cancer Resea* **2**, 739 (1942).
158. Pollack, M. A., Taylor, A., Woods, A., Thompson, R. C., and Williams, R. *ibid.*, **2**, 748 (1942).
*159. Potter, V. R., *Medicine*, **19**, 441 (1940).
160. Potter, V. R., *J. Biol. Chem.*, **134**, 417 (1940).
161. Potter, V. R., *ibid.*, **141**, 775 (1941).
162. Potter, V. R., *Cancer Research*, **2**, 688 (1942).
163. Potter, V. R., *ibid.*, **3**, 358 (1943).
164. Potter, V. R., *J. Am. Dietet. Assoc.*, **19**, 488 (1943).
165. Potter, V. R., *unpublished.*
*166. Potter, V. R., Gibson Island Research Conference on Chemical Growth moters. *Unpublished*, 1943.
167. Potter, V. R., and Albaum, H. G., *J. Gen. Physiol.*, **26**, 443 (1943).
168. Potter, V. R., and DuBois, K. P., *J. Biol. Chem.*, **142**, 417 (1942).
169. Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, **26**, 391 (1943).
170. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
171. Potter, V. R., and Liebl, G. J., *unpublished.*
172. Potter, V. R. and Schneider, W. C., *unpublished.*
173. Reid, M. E., *Physiol. Revs.*, **23**, 76 (1943).
174. Reimann, S. P., and Hall, E. M., *Arch. Path.*, **22**, 55 (1936).
175. Reimann, S. P., *Growth*, **6**, 273 (1942).
176. Rigler, R., *Arch. Exptl. Path. Pharm.*, **167**, 54 (1932).
177. Robertson, W. v. B., and Kahler, H., *J. Natl. Cancer Inst.*, **2**, 595 (1942).
178. Rosenthal, O., *Biochem. J.*, **31**, 1710 (1937).
179. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **149**, 437 (1943).
179a. Rosenthal, O., and Drabkin, D. L., *ibid.*, **150**, 131 (1943).

180.   Rosenthal, O., and Lasnitzki, A., *Biochem. Z.*, **196**, 340 (1928).
180a.   Roskelley, R. C., Mayer, N., Horwitt, B. N., and Salter, W. T., *J. Clin. Investigation*, **22**, 743 (1943).
181.   Rous, P., and Kidd, J. G., *J. Exptl. Med.*, **73**, 365 (1941).
*182.   Rusch, H. P., *Physiol. Revs.*, **24**, 177 (1944).
*183.   Sacks, J., *Physiol. Revs.*, **21**, 217 (1941).
184.   Sacks, J., and Altshuler, C. H., *Am. J. Physiol.*, **137**, 750 (1942).
185.   Scharles, F. H., Baker, M. D., and Salter, W. T., *Biochem. J.*, **29**, 1927 (1935).
186.   Scharles, F. H., Baker, M. D., and Salter, W. T., *Am. J. Cancer*, **25**, 122 (1935).
*187.   Schlenk, F., in *A Symposium on Respiratory Enzymes.* Univ. Wisconsin Press, Madison, 1942, p. 104.
188.   Schlenk, F., and Gingrich, W., *J. Biol. Chem.*, **143**, 295 (1942).
189.   Schneider, W. C., and Potter, V. R., *Cancer Research*, **3**, 353 (1943).
190.   Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
191.   Schneider, W. C., and Potter, V. R., *unpublished.*
192.   Schultze, M. O., *J. Biol. Chem.*, **129**, 729 (1939).
193.   Shack, J., *J. Natl. Cancer Inst.*, **3**, 389 (1943).
194.   Sharp, D. G., Taylor, A. R., Beard, D., Finkelstein, H., and Beard, J. W., *Science*, **92**, 359 (1940).
195.   Shear, M. J., *Am. J. Cancer*, **33**, 499 (1938).
196.   Shelley, W. B., Code, C. F., and Visscher, M. B., *Am. J. Physiol.*, **138**, 652 (1943).
*197.   Shorr, E., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 323 (1939).
198.   Smadel, J. E., Lavin, G. I., and Dubos, R. J., *J. Exptl. Med.*, **71**, 373 (1940).
199.   Snell, E. E., and Strong, F. M., *Ind. Eng. Chem., Anal. Ed.*, **11**, 346 (1939).
*200.   Soskin, S., *Arch. Internal Med.*, **71**, 219 (1943).
*201.   Stannard, J. N., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 394 (1939).
*202.   Stern, K. G., *ibid.*, **7**, 312 (1939).
203.   Stern, K. G., and Duran-Reynals, F., *Science*, **89**, 609 (1939).
204.   Stevenson, E. S., Dobriner, K., and Rhoads, C. P., *Cancer Research*, **2**, 160 (1942).
205.   Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).
206.   Straub, F. B., *Biochem. J.*, **33**, 787 (1939).
207.   Straub, F. B., *Enzymologia*, **9**, 148 (1941).
208.   Swingle, K. F., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 581 (1942).
*209.   Szent-Györgyi, A., *Studies on Biological Oxidation.* Stechert, New York, 1937.
210.   Taylor, A., Pollack, M. A., Hofer, M. J., and Williams, R. J., *Cancer Research*, **2**, 744 (1942).
211.   Taylor, A., Pollack, M. A., Hofer, M. J., and Williams, R. J., *ibid.*, **2**, 752 (1942).
212.   Taylor, A., Pollack, M. A., and Williams, R. J., *Science*, **96**, 322 (1942).
213.   Theorell, H., *Biochem. Z.*, **285**, 207 (1936).
214.   *The University of Texas Publication*, No. 4137, Oct. 1, 1941.
215.   *Ibid.*, No. 4237, Oct. 1, 1942.
216.   Tsuzuki, K., *J. Biochem. Japan*, **23**, 421 (1936).
*217.   Umbreit, W. W., Gibson Island Research Conference on Chemical Growth Promoters. *Unpublished*, 1943.

218. Vogler, K. G., LePage, G. A., and Umbreit, W. W., *J. Gen. Physiol.*, **26,** 89 (1942).
219. Vogler, K. G., and Umbreit, W. W., *ibid.*, **26,** 157 (1942).
220. Warburg, O., *Biochem. Z.*, **160,** 307 (1925).
221. Warburg, O., and Christian, W., *ibid.*, **287,** 291 (1936).
222. Warburg, O., and Christian, W., *ibid.*, **292,** 287 (1937).
223. Warburg, O., and Christian, W., *ibid.*, **298,** 150 (1938).
224. Warburg, O., and Christian, W., *ibid.*, **303,** 40 (1939).
225. Warburg, O., Christian, W., and Griese, A., *ibid.*, **282,** 157 (1935).
226. Warburg, O., Posener, K., and Negelein E., *ibid.*, **152,** 309 (1924).
227. West, P. M., and Woglom, W. H., *Cancer Research*, **2,** 324 (1942).
228. Weise, E., *Arch. Exptl. Path. Pharm.*, **176,** 367 (1934).
229. White, J., and White, A., *J. Biol. Chem.*, **131,** 149 (1939).
230. Wood, J. L., and Fieser, L. F., *J. Am. Chem. Soc.*, **62,** 2674 (1940).
231. Zittle, C. A., and Zitin, B., *J. Biol. Chem.*, **144,** 99 (1942).

THE INFLUENCE OF HORMONES
ON ENZYMATIC REACTIONS

By

H. JENSEN AND LEON E. TENENBAUM

Kalamazoo, Michigan

CONTENTS

I. Introduction

Metabolism is controlled by the physiological coordination of various active agents in the body. The active agents may be classified as hormones, enzymes, and vitamins. A close physiological interrelationship between the different principles seems to exist.

No definite information is as yet available regarding the role of hormones in enzymatic reactions. Most investigations have been confined to a study of the influence of hormones on the enzymatic activity of certain organ or tissue preparations. It is with these studies that this review is mainly concerned. Caution in the interpretation of results obtained by *in vitro* experiments under various experimental conditions must be observed (6).

All animal hormones which have been isolated up to the present are either steroid or protein and amino acid derivatives. It may be mentioned that synthesis of a hormone apparently takes place only in the

intact cells of the specific tissue of the endocrine organ. Chaikoff and his associates demonstrated a rapid formation of diiodotyrosine and thyroxine *in vivo* (47) as well as *in vitro* by intact but not by homogenized thyroid tissue (41).

It is expedient to arrange the subject matter of this chapter under the headings of the different hormones to be discussed in relation to their possible influence on various enzymatic reactions.

II. The Pituitary Hormones

1. Posterior Pituitary

The secretion of the pars neuralis of the pituitary (vasopressor and oxytocic principles*) has as yet not been studied concerning its effect on certain enzymatic reactions. It is known that it produces a breakdown of liver glycogen to glucose (16). Fostvedt (19) reported that the melanophore principle increases tyrosinase activity; the presence of either the oxytocic or the pressor principle apparently inhibits this activation.

2. Anterior Pituitary

With probably the exception of the growth factor, all the other established anterior pituitary principles exert their effects by stimulation of other endocrine organs (adrenal cortex, thyroid, gonads, and mammary gland). The nature of the stimulation may be twofold:

1.   Causing a release of the active principle of the organ.

2.   Enhancing formation of the active principle, the synthesis of which is brought about by certain intercellular enzymes.

Practically nothing is known about those enzymes which play a role in the synthesis of hormones. For instance, the thyroid functions are under the control of the anterior pituitary (thyrotrophic principle) as to the rate of iodine uptake and the conversion of diiodotyrosine to thyroxine (43, 44).

Hypophysectomy reduces tissue respiration, which can be restored on injection of thyrotrophic pituitary preparations (39). Fraenkel-Conrat, Simpson, and Evans (20) reported that administration of the adrenocorticotrophic principle causes an increase in liver arginase, while the growth factor produces a decrease.

---

* Whether the physiological effects of the secretion of the pars neuralis of the pituitary are exerted by a single principle or two distinct factors is a question still under debate.

III. Insulin

Insulin, the internal secretion of the pancreas, is indispensable for the normal maintenance of carbohydrate metabolism in mammals. The following physiological functions may be attributed to insulin:

1. Acceleration of glucose oxidation in the tissues.

2. Increase in the rate at which glucose is converted to glycogen in the muscle and liver; it has been shown that oxidation of glucose and formation of muscle glycogen can take place in the absence of insulin (depancreatized animals).

3. Inhibition of glycogenolysis in the liver.

4. Suppression of protein and fat catabolism, leading to a decrease of both gluconeogenesis from amino acids and ketone formation from fatty acids in the liver.

Various enzyme systems are concerned with these different metabolic changes. The rate at which these processes take place in the cells of various tissues is apparently influenced by insulin.

Earlier attempts to demonstrate an *in vitro* effect of insulin, carried out with rather impure insulin preparations, have not given uniform results. A discussion of these papers is omitted, and for information on this subject the reader is referred to references (22, 27, and 29). In studies of an *in vitro* effect of insulin, one must take into account the possibility that the observed response may perhaps be due to nonspecific protein or certain metals (zinc) present in the insulin preparation and not to the hormone itself.

A low R.Q. has been observed in isolated slices of liver, kidney, and brain from diabetic animals (17, 28). In 1938, Krebs and Eggleston (34) observed that insulin increases the oxygen uptake of a suspension of minced pigeon breast muscle in phosphate buffer in the presence of citrate. Stadie, Zapp, and Lukens (61) confirmed this effect but, like Shorr and Barker (58), found it to be smaller than had originally been observed, and also found it to be independent of the simultaneous addition of citrate. Stare and Baumann (63) observed that the effect of insulin was more pronounced when muscles from pancreatectomized pigeons were used. Rice and Evans (50) concluded from the results of their experiments with minced pigeon breast muscle that insulin is concerned in maintaining functional integrity of either one or both of the enzyme systems involved in the reactions of fumaric and pyruvic acids or of oxaloacetic and pyruvic acids.

Gemmill (22, 23, 24) has published a series of papers demonstrating an effect of insulin on glycogen deposition in isolated mammalian muscle; rat's diaphragm was suspended in oxygenated Ringer's solution containing

glucose. According to Gemmill, the *in vitro* deposition of glycogen in muscle by insulin requires glucose as its substrate. Insulin, while accelerating the utilization of glucose, does not increase the consumption of oxygen by the isolated muscle; therefore, the main function of insulin is to aid in the deposition of glycogen in muscle from glucose in the medium. According to Hechter, Levine, and Soskin (25), the glycogen deposition in rat diaphragm *in vitro* varies directly with the concentration of the glucose in the medium. Insulin catalyzes this process greatly at low glucose concentration but very slightly at high concentration. Apparently, insulin is not necessary for glycogen formation but increases the rate of the process in the tissue. The reaction, glucose ⇄ glycogen, can be reproduced in cell-free enzyme systems without the intervention of insulin. Cori (13, 14) has advanced the hypothesis that insulin is not an essential component of those enzyme systems converting glucose to glycogen (muscle and liver) or oxidizing glucose, and that its effect of accelerating or inhibiting these enzymatic reactions is dependent on a more or less intact cell structure.

Seckel (55), studying the influence of insulin on the glycogenolysis of surviving rat liver slices, concluded that the breakdown of liver glycogen to glucose is inhibited by insulin. Taubenhaus, Levine, and Soskin (66) arrived at a similar conclusion from their experimental results.

Bach and Holmes (2) have reported that the transformation of certain amino acids into carbohydrate, observed in excised liver slices *in vitro*, was inhibited by insulin, and that this inhibition was accompanied by a reduction of urea formation. Stadie, Lukens, and Zapp (60) have extended the experiments of Bach and Holmes (2). They conclude from their experimental findings, obtained with liver slices of rats, that insulin inhibits the deamination of the unnatural or *d*-isomers of amino acids. The corresponding natural or *l*-isomers were unaffected. In this connection it may be pointed out that it has been found that natural or *l*-amino acid oxidase is apparently effective only in the intact cell. Furthermore, in the complete absence of insulin (cats 48 hours after pancreatectomy), deamination by liver slices was found to be much greater than normal. These observations seem to support the accepted belief that insulin inhibits gluconeogenesis. For further information on the relation of hormones to carbohydrate metabolism *in vitro*, the reader is referred to a review article by Shorr (57).

Stadie, Zapp, and Lukens (62) have studied ketogenesis and antiketogenesis *in vitro* with liver slices from fasted normal and diabetic cats. They found that the liver slices from diabetic animals produced ketones at a higher rate than those from the normal animal. Insulin, in the presence

of fructose, fumarate, and d-lactate, inhibits the formation of ketones by the diabetic liver. This effect was enhanced by prolonged equilibration of slices with insulin-containing media. These observations support the view that insulin inhibits the formation of ketone bodies.

The problem of the interaction of the many different factors controlling fat metabolism in the liver grows increasingly more complicated, and further work is necessary before a complete picture can be obtained. Further advances depend on *in vitro* studies of the enyzme system involved in the metabolism of fats and the influence of certain hormones on these enzymatic reactions. It may be pointed out that the liver is, in all probability, the organ through which the influence of the endocrine system on metabolism is mainly exerted. It is this organ that is the site of glucose formation and probably of the major processes of fat metabolism. The use of liver slices in the study of the influence of hormones on certain metabolic reactions, controlled by enzymes, is therefore of special interest.

The following sections discuss the influence on enzymatic reactions of those hormones whose secretions are known to be controlled by the anterior pituitary. As yet, no definite proof has been obtained to indicate that the secretion of insulin is under direct influence of the anterior pituitary.

IV. Thyroid Hormone

The nature of the hormone secreted by the thyroid is still under investigation. It has been recognized that it is not thyroxine (40). Evidence has been obtained that thyroglobulin does not occur in the blood (36, 54, 64). Canzanelli, Guild, and Rapport (5) found that the Q_{O_2} of slices of kidney, testis, and heart muscle from the guinea pig, when tested *in vitro*, was increased by thyroglobulin, but not by thyroxine. In passing, mention may be made that the possibility of synthesis of thyroxine by tissues other than the thyroid is suggested by the observation that the addition of small amounts of iodine to a low iodine diet appeared to benefit thyroidectomized rats (7), and by the finding that extrathyroid thyroxine synthesis could be demonstrated through the isolation of radioactive thyroxine following the administration of radioactive iodine to thyroidectomized rats (42).

A great deal of work has been carried out on the influence of the thyroid hormone on various respiratory systems. Many investigators have reported increases in the oxygen uptake of surviving tissues after treating animals with thyroxine or thyroid preparations. For earlier work on this subject the reader is referred to a paper by Rossiter (51), reporting that, in the presence of glucose, sodium pyruvate, or sodium succinate, brain brei from thyroid and vitamin B_1 treated rats has a higher oxygen uptake

than brei from controls which received vitamin B_1 only. *In vitro* addition of thyroglobulin causes an increase in oxygen uptake of dispersion preparations (brei) of rat brain with both glucose and sodium pyruvate. *In vitro* addition of thyroxine does not cause any increase under these conditions. On the other hand, Spirtes (59) found that the feeding of desiccated thyroid to guinea pigs caused no apparent increase in oxygen consumption of slices of brain tissue prepared from these animals, although that of kidney and of liver was increased 28% and 52%, respectively.

Klein (31, 32) reported that there is a decrease in the content of *d*-amino acid oxidase in the tissues of thyroidectomized rats, and an increase in content in the tissues of animals maintained on an adequate diet supplemented with thyroid tissue. The increased activity in the latter instance is not due to an increase in the content of the prosthetic group (flavin-adenine-dinucleotide) of the enzyme, but is probably associated with an increased concentration of the specific protein portion of the enzyme system.

An increase in the phosphatase content of bone was observed after the administration of thyroxine (74). It has been reported that livers of female rats given thyroxine contained quantities of arginase comparable to those found after feeding diets high in protein or after long fasting. However, administration of thyroxine to male rats was not associated with any change in liver arginase (37).

V. Parathyroid Hormone

In vitro studies on the influence of parathyroid hormone on kidney phosphatase indicate that activation occurs. However, the effect is not specific, since egg albumin and serum albumin were found to produce a similar response (75). A drop in the phosphatase content of bone has been observed after the administration of parathyroid extract (73).

VI. Epinephrine

It has been established from experiments on intact animals and on isolated organs that epinephrine accelerates the breakdown of glycogen in the liver to glucose and of glycogen in the muscle to lactic acid (11). Concerning the enzyme systems involved in these reactions, the reader is referred to review articles by Cori (12, 13, 14).

Hegnauer and Cori (26) found that, when resting frog muscle is kept anaerobically in Ringer's solution, the rate of breakdown of glycogen is markedly increased by the addition of epinephrine, and hexose monophosphate accumulates in amounts equivalent to the inorganic phosphate

which disappeared. Bendall and Lehmann (3) showed by *in vitro* experiments with liver slices from rabbits and rats that epinephrine causes a breakdown of liver glycogen. In the presence of glucose, the synthesis of glycogen is at first inhibited and then increased. According to Cori (12), glycogen breakdown in the liver and muscle is initiated by the same enzyme (phosphorylase), and it is probable that the point of action of epinephrine is the same in both tissues. It should be pointed out that the accelerating effect of epinephrine on phosphorylase activity has thus far been observed only in intact cells. In enzyme solutions, no effect of epinephrine on phosphorylase activity has as yet been demonstrated. It is possible that this hormone exerts its effect by increasing the concentration of the active enzyme in the cell.

VII. The Adrenal Cortical Hormones

In recent years it has been shown that certain principles, secreted by the adrenal cortex, induce suppression of the utilization of glucose, increase the rate of formation of glucose from protein, and influence fat and salt metabolism (38, 67). For the chemistry of the various active principles isolated from the adrenal cortex, the reader's attention is called to references (48 and 49).

In a series of publications, Verzár and coworkers (69, 70) have presented experimental evidence which they interpret as indicating that the cortex of the adrenal gland has some specific control over the phosphorylations involved in the utilization of glucose, fat, and vitamins B_1 and B_2. However, more recent work has failed to substantiate most of the claims of Verzár and has shown that the function of the adrenal cortex is not primarily concerned with phosphorylation. It has been demonstrated for vitamins B_1 and B_2 that their phosphorylation does not depend on an adrenal cortical factor (4, 8, 18, 45, 46). Absorption of glucose (via phosphorylation) is apparently not under the direct influence of the adrenal cortex. Clark and MacKay (9) found a decrease in the rate of absorption of glucose in adrenalectomized rats when the salt balance was upset. However, when the normal salt balance was preserved by proper salt therapy, the absorption of glucose was not diminished in the operated animals. Their findings confirm earlier reports of other investigators (1, 15). These results would seem to show that, although the role of the adrenal cortex may be of importance in glucose absorption, it is only an indirect one and acts by maintaining a normal salt balance, thus preventing hemoconcentration and reduction in blood flow to the gut, leading secondarily to a slowing up of absorption. Stillman, Entenman, Anderson, and

Chaikoff (65) found direct proof that phosphorylation of fat, as measured by the incorporation of administered radioactive phosphorus into the phospholipid molecule, is not interrupted in the liver and small intestine of the adrenalectomized rat. Verzár and Montigel (71, 72) reported that the lowered rate of phosphorylation of glycogen in minced muscle from adrenalectomized animals, observed in *in vitro* experiments, could be restored to normal by the addition of desoxycorticosterone. They claim that the effect is specific for desoxycorticosterone and is the first instance demonstrating an *in vitro* response of the principle.

Kutscher and Wüst (35) observed a pronounced decrease in the phosphatase activity in the small intestines and kidneys of adrenalectomized guinea pigs. Williams and Watson (73) have made a study of the effect of various adrenal cortical principles on the phosphatase content of rats' femurs. Adrenal cortical extract and corticosterone were found to cause a decrease in the content of the enzyme, while desoxycorticosterone acetate produced an increase.

Seckel (56) reported that adrenal cortical extract will inhibit the breakdown of liver glycogen in rat liver slices. According to Tipton (68), adrenal insufficiency in rats results in a significant depression of respiration of liver and kidney slices, developing about a week after the operation and being associated with considerable diminution in the rate of oxidation of pyruvate and succinate by liver tissue. Corey and Britton (10) found that the perfusion of cat liver with glucose–Ringer–gum solution containing adrenal cortical extract effected an increase of from 50 to 100% in the glycogen content in fifteen minutes; however, desoxycorticosterone was found to be ineffective. According to Koepf, Horn, Gemmill, and Thorn (33), adrenal cortical extract increases glycogen synthesis in rat liver slices from pyruvate and d-lactate but not from d,l-alanine and d-glutamate.

Russell and Wilhelmi (52) reported that kidney tissue slices from adrenalectomized rats have a lower rate of oxygen uptake than normal kidney slices both in the absence and in the presence of substrates. The rate of ammonia production from amino acids is also less than normal. These defects can be repaired by the administration of adrenal cortical extract or desoxycorticosterone to the adrenalectomized animals. Later work by the same investigators (53), indicates that, while the rate of glucose utilization and the rate of carbohydrate formation from succinic and pyruvic acids in kidney slices are unaffected by adrenalectomy, there is a significantly lower rate of carbohydrate formation from d,l-alanine and $l(+)$-glutamic acid. These findings indicate that an important factor limiting the rate of gluconeogenesis after adrenalectomy is the rate of deamination of amino acids.

Karady, Rose, and Browne (30) found that adrenalectomy in rats produced a diminution of the histaminase content of the lung, and that the histaminase activity could be restored to normal values by the administration of an extract of the adrenal cortex. Fraenkel-Conrat, Simpson, and Evans (21) reported that adrenalectomy caused a marked decrease of the arginase activity of rat livers. Administration of those adrenal cortical principles containing an oxygen at C_{11} was found to produce an increase of the liver arginase in normal and adrenalectomized animals.

VIII. Sex Hormones

Estrogens and androgens no doubt affect metabolism either directly in the tissues or indirectly by stimulating or inhibiting the rate of secretion of other endocrine organs. *In vitro* studies on the effect of this group of endocrine principles on a given enzyme system have as yet not been reported.

IX. Conclusion

It has been the aim of this review to summarize the results of various investigations on the influence of hormones on enzymatic reactions. It is apparent that our present knowledge of the mechanism by which the various endocrine principles may affect enzymatic reactions is rather meager and does not permit any definite conclusions. It should be pointed out that results obtained by *in vitro* experiments may be artifacts, and may be without significance in the living animal. Caution must also be exercised against making generalizations from results obtained with only one species of animal.

It is possible that hormones do not participate in the actual enzymatic processes, *i. e.*, are not essential components of a given enzyme system. They may accelerate or inhibit certain enzymatic reactions or may cause an increased concentration of a given enzyme in the tissue upon which they act. It appears that the action of hormones on the rate of enzymatic reactions takes place only in the intact cell. It is known that the activity of certain enzymes is dependent on cellular integrity.

The importance of future investigations in this field is obvious. Results obtained should help in securing a better understanding of the role which hormones play in the various processes of metabolism.

Bibliography

1. Althausen, T. L., Anderson, E. M., and Stockholm, M., *Proc. Soc. Exptl. Biol. Med.*, **40,** 342 (1939).

2. Bach, S. J., and Holmes, E. G., *Biochem. J.*, **31**, 89 (1937).

3. Bendall, J. R., and Lehmann, H., *Nature*, **148**, 538 (1941).

4. Bruce, H. M., and Wien, R., *J. Physiol.*, **98**, 375 (1940).

5. Canzanelli, A., Guild, R., and Rapport, D., *Endocrinology*, **25**, 707 (1939).

6. Chambers, W. H., Shorr, E., and Barker, S. B., *Ann. Rev. Physiol.*, **4**, 158 (1942).

7. Chapman, A., *Endocrinology*, **27**, 686 (1941).

8. Clark, W. G., *ibid.*, **28**, 545 (1941).

9. Clark, W. G., and MacKay, E. M., *Am. J. Physiol.*, **137**, 104 (1942).

10. Corey, E. L., and Britton, S. W., *ibid.*, **131**, 783 (1940).

11. Cori, C. F., *Physiol. Revs.*, **11**, 143 (1931).

12. Cori, C. F., *Endocrinology*, **26**, 285 (1940).

13. Cori, C. F., *Biol. Symposia*, **5**, 131 (1941).

14. Cori, C. F., *Symposium on Respiratory Enzymes.* Univ. of Wisconsin Press, Madison, 1942, p. 175.

15. Deuel, H. J., Jr., Hallman, L. F., Murray, S., and Samuels, L. T., *J. Biol. Chem.*, **119**, 607 (1937).

16. Ellsworth, H. C., *J. Pharmacol.*, **56**, 417 (1936).

17. Fazekas, J. F., Baker, Z., and Himwich, H. E., *Am. J. Physiol.*, **123**, P62 (1938).

18. Ferrebee, J. W., *J. Biol. Chem.*, **136**, 719 (1940).

19. Fostvedt, G. A., *Endocrinology*, **27**, 100 (1940).

20. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *Am. J. Physiol.*, **138**, 439 (1943).

21. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **147**, 99 (1943).

22. Gemmill, C. L., *Bull. Johns Hopkins Hosp.*, **66**, 232 (1940).

23. Gemmill, C. L., *ibid.*, **68**, 329 (1941).

24. Gemmill, C. L., and Hamman, L., Jr., *ibid.*, **68**, 50 (1941).

25. Hechter, O., Levine, R., and Soskin, S., *Proc. Soc. Exptl. Biol. Med.*, **46**, 390 (1941).

26. Hegnauer, A. H., and Cori, G. T., *J. Biol. Chem.*, **105**, 691 (1934).

27. Hill, D. W., and Howitt, F., *Insulin, Its Production, Purification, and Physiological Action.* Chemical Pub. Co., New York, 1935; Hutchinson, London, 1936.

28. Himwich, H. E., and Fazekas, J. F., *Proc. Soc. Exptl. Biol. Med.*, **38**, 137 (1938).

29. Jensen, H., *Insulin, Its Chemistry and Physiology.* Commonwealth Fund, New York, 1938.

30. Karady, S., Rose, B., and Browne, J. S. L., *Am. J. Physiol.*, **130**, 539 (1940).

31. Klein, J. R., *J. Biol. Chem.*, **128**, 659 (1939).

32. Klein, J. R., *ibid.*, **131**, 139 (1939).

33. Koepf, G. F., Horn, H. W., Gemmill, C. L., and Thorn, G. W., *Am. J. Physiol.*, **135**, 175 (1941).

34. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **32**, 913 (1938).

35. Kutscher, W., and Wüst, H., *Naturwissenschaften*, **29**, 319 (1941).

36. Lerman, J., *J. Clin. Investigation*, **19**, 555 (1940).

37. Lightbody, H. D., Witt, E., and Kleinman, A., *Proc. Soc. Exptl. Biol. Med.*, **46**, 472 (1941).

38. Long, C. N. H., Katzin, B., and Fry, E., *Endocrinology*, **26**, 309 (1940).

39. Macleod, L. D., and Reiss, M., *Biochem. J.*, **34**, 820 (1940).

40. McClendon, J. F., and Foster, W. C., *Endocrinology*, **29**, 653 (1941).

41. Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, **144**, 565 (1942).

42. Morton, M. E., Chaikoff, I. L., Reinhardt, W. O., and Anderson, E., *ibid.*, **147**, 757 (1943).

43. Morton, M. E., Perlman, I., Anderson, E., and Chaikoff, I. L., *Endocrinology*, **30**, 495 (1942).

44. Morton, M. E., Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **140**, 603 (1941).

45. Nelson, D., *Am. J. Physiol.*, **129**, P429 (1940).

46. Ochoa, S., and Rossiter. R. J., *J. Physiol.*, **97**, 1P (1940).

47. Perlman, I., Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, **137**, 449 (1941).

48. Pfiffner, J. J., *Advances in Enzymology*, Vol. II. Interscience, New York, 1942, p. 325.

49. Reichstein, T., and Shoppee, C. W., *Vitamins and Hormones*. Vol. I, Academic Press, New York, 1943, p. 345.

50. Rice, L., and Evans, E. A., Jr., *Science*, **97**, 470 (1943).

51. Rossiter, R. J., *J. Endocrinol.*, **2**, 165 (1940).

52. Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, **137**, 713 (1941).

53. Russell, J. A., and Wilhelmi, A. E., *ibid.*, **140**, 747 (1941).

54. Salter, W. T., Bassett, A. M., and Sappington, T. S., *Am. J. Med. Sci.*, **202**, 527 (1941).

55. Seckel, H. P. G., *Endocrinology*, **23**, 760 (1938).

56. Seckel, H. P. G., *ibid.*, **26**, 97 (1940).

57. Shorr, E., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 323 (1939).

58. Shorr, E., and Barker, S. B., *Biochem. J.*, **33**, 1798 (1939).

59. Spirtes, M. A., *Proc. Soc. Exptl. Biol. Med.*, **46**, 279 (1941).

60. Stadie, W. C., Lukens, F. D. W., and Zapp, J. A., Jr., *J. Biol. Chem.*, **132**, 393 (1940).

61. Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *ibid.*, **132**, 411 (1940).

62. Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *ibid.*, **132**, 423 (1940).

63. Stare, F. J., and Baumann, C. A., *ibid.*, **133**, 453 (1940).

64. Stellar, L. I., and Olken, H. G., *Endocrinology*, **27**, 614 (1940).

65. Stillman, N., Entenman, C., Anderson, E., and Chaikoff, I. L., *ibid.*, **31**, 481 (1942).

66. Taubenhaus, M., Levine, R., and Soskin, S., *Proc. Soc. Exptl., Biol. Med.*, **42**, 693 (1939).

67. Thorn, G. W., Koepf, G. F., Lewis, R. A., and Olsen, E. F., *J. Clin. Investigation*, **19**, 813 (1940).

68. Tipton, S. R., *Am. J. Physiol.*, **132**, 74 (1940).

69. Verzár, F., *Die Funktion der Nebennierenrinde*. Schwabe, Basel, 1939.

70. Verzár, F., and McDougall, E. J., *Absorption from the Intestine*. Longmans, Green, London, 1936.

71. Verzár, F., and Montigel, C., *Helv. Chim. Acta*, **25**, 22 (1942).

72. Verzár, F., and Montigel, C., *Nature*, **149**, 49 (1942).

73. Williams, H. L., and Watson, E. M., *Endocrinology*, **29**, 250 (1941).

74. Williams, H. L., and Watson, E. M., *ibid.*, **29**, 258 (1941).

75. Wood, T. R., and Ross, W. F., *J. Am. Chem. Soc.*, **64**, 2759 (1942).

THE ABSORPTION SPECTRA OF VITAMINS, HORMONES, AND ENZYMES

By

WALLACE R. BRODE

Columbus, Ohio

CONTENTS

269

I. Introduction

The recent publication (1942) of the extensively revised edition of Morton's book (52) on the application of absorption spectra to the study of vitamins, hormones and coenzymes provides a current source of information on the topic which covers the field far more adequately than can be done in this brief survey.   For more detailed information on most of the topics discussed in this chapter, the reader should consult Morton's text.
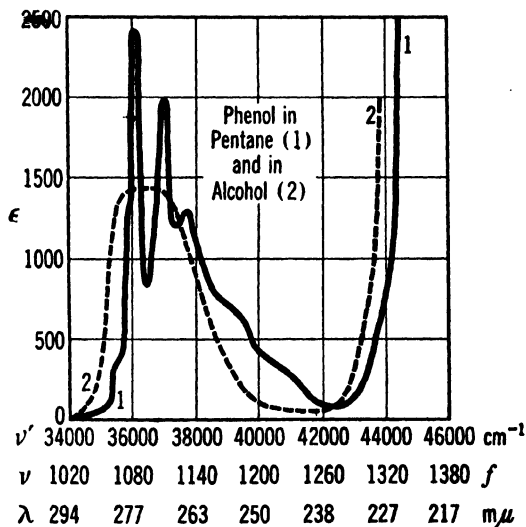
Fig. 1.—Absorption spectra of phenol in alcohol and pentane show enhanced resolution in a non-polar solvent (pentane).   Data are indicated in molecular extinction (ϵ) as ordinate and as abscissa in wave length (λ) in angstroms (Å), and in wave number (ν' or $1/\lambda$), in reciprocal centimeters (cm.$^{-1}$), and in frequency (ν), in fresnels (f).   It is not customary to use more than one system of ordinate or abscissa at a time, but the three abscissa notations have been shown here to indicate their relation to each other.

For information concerning methods of recording absorption spectra and the significance and interpretation of absorption spectra data the reader is referred to the revised edition (1943) of *Chemical Spectroscopy* (4) by the author of this chapter.

As an example of the methods used in recording absorption spectra data, Figure 1 shows the absorption curves of phenol in pentane and alcohol.   The abscissae are usually

expressed in wave length (λ) in units of millimicrons (mμ) (10^{-9} meters) or angstroms (Å) (10^{-10} meters). In studies involving the analysis of absorption band structure, the data are sometimes expressed in wave number (ν') in units of cm^{-1}, or frequency (ν) in fresnel unit (f). The interrelation of these systems is indicated as ($1/\lambda = \nu' = \nu/c$) where c equals the speed of light.

The ordinate values indicate absorption intensity and are usually expressed in extinction coefficient (k) or extinction (E), by

$$E = kcd = \log I_0/I$$

where c = concentration in g. per ml., d = cell thickness in cm., and I_0 and I are the incident and transmitted light intensities. Two other units widely used in biochemical analyses to indicate absorption values are molecular extinction (ϵ), in which c is expressed in gram moles per liter and $E_d^\%$, in which the extinction is recorded for a given concentration in $\%$ and thickness (d) in cm.

As an example of this latter method, it might be pointed out that the determination of vitamin A concentration is based on $E_{1cm.}^{1\%}$ 325 mμ = 1880, which signifies that, at the wave length of 325 mμ (the maxima of the absorption band in this case), the extinction for a 1$\%$ solution of vitamin A in a 1-cm. cell would be 1880. Actually, one cannot read extinction this high; and this value is obtained by calculation from thinner cells and more dilute solutions. The vitamin A content of an unknown oil or preparation is determined from the extinction value at 325 mμ of known concentrations and cell thickness. If the observed value on an unknown oil for a 2$\%$ solution in a 2.5-cm. cell is $E = 2.6$ then the E value for a 1$\%$,1-cm. cell will be $E_{1cm.}^{1\%} = 2.6/(2.5 \times 2.0) = 0.52$.

Based on biological tests of β-carotene, a 1$\%$ solution of pure vitamin A has been assigned a vitamin potency of 3.3×10^{-4} I.U. (International Units) per ml. The extinction of pure vitamin A, $E_{1cm.}^{1\%}$ 325 mμ is 1880. Hence, the potency of the above sample is $(0.52/1880) \times 3.3 \times 10^{-4} = 9.1$ I.U.

The use of absorption spectra in the study of vitamins, hormones, and coenzymes is a mechanism or tool for determining concentration, structure, and identity. The application of such methods will be discussed under the various compounds to which these methods of analysis are applied.

II. The Absorption Phenomenon

In this general discussion of the significance of method and application, it will be essential to consider the fundamentals underlying the absorption phenomenon. The absorption of light by compounds in the visual and ultraviolet region of the spectrum results from certain types of intermolecular vibration. From the chemist's point of view, such intermolecular vibration which produces absorption is known as resonance (42). Resonance does not indicate a single structure, rather the existence of two or more electronic structures in dynamic equilibrium—for example, the ketone

structure may be written normally R—C—R, whereas the resonating structure is represented as the summation of the possible structures, as:

$$\begin{array}{ccccc}
:\overset{..}{\overset{-}{\text{O}}}: & & :\text{O}: & & :\overset{..}{\overset{+}{\text{O}}}: \\
\text{R}:\overset{..}{\underset{+}{\text{C}}}:\text{R} & \underset{\longleftarrow}{\overrightarrow{}} & \text{R}:\overset{::}{\text{C}}:\text{R} & \underset{\longleftarrow}{\overrightarrow{}} & \text{R}:\underset{..}{\text{C}}:\text{R}
\end{array}$$
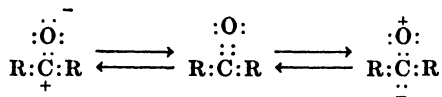
One of the best examples of resonance is that produced in unsaturated formation such as the ethenylene linkage (—CH=CH—) which in its simplest form, ethylene, shows a band at 195 mμ. The more complex organic resonators are generally made up of combinations of the simpler resonators, such as $\diagup \text{C}=\text{C} \diagdown$, $\diagup \text{C}=\text{O}$, $\diagup \text{C}=\text{N}$, —N=O.

In the classification of colored or absorbing systems, it is customary to ascribe to a certain part of the molecule the resonating effect which is considered responsible for the color; and such a group is known as the chromophore (4). The groups given in Table I are some of those generally included in a list of organic chromophores.

TABLE I

TYPICAL ORGANIC CHROMOPHORES

Name of group	Formula	Electronic forms
Ethenylene	$\diagdown\text{C}=\text{C}\diagup$	$\diagdown :\overset{.}{\text{C}}::\overset{.}{\text{C}}: \diagup$, $\diagdown \overset{.}{\underset{+}{\text{C}}} \ :\underset{..}{\text{C}}: \diagup$
Carbonyl	$\diagdown\text{C}=\text{O}$	$\diagdown :\overset{..}{\text{C}} \ \overset{..}{\underset{..}{\text{O}}}:, \ \diagdown :\overset{.}{\underset{..}{\text{C}}}::\overset{..}{\underset{+}{\text{O}}}:, \ \diagdown \overset{.}{\underset{+}{\text{C}}} \ :\overset{..}{\underset{..}{\text{O}}}:$
Azo	—N=N—	$\overset{..}{\text{N}}::\overset{..}{\text{N}}—, \ —\overset{..}{\underset{..}{\text{N}}}::\overset{..}{\underset{+}{\text{N}}}—$
Nitroso	—N=O	$—\overset{..}{\text{N}}::\overset{..}{\underset{..}{\text{O}}}:, \ —\overset{..}{\underset{..}{\text{N}}}: \ \overset{..}{\underset{+}{\text{O}}}:, \ —\overset{..}{\underset{+}{\text{N}}} \ :\overset{..}{\underset{..}{\text{O}}}:$

Chromophoric changes may be resolved into two effects, intensity and frequency shifts, which are described as follows:

(a) *Hyperchrome.* An increase in the extinction value of the absorption band. *The intensity of the color increases.*

(b) *Hypochrome.*   A decrease in the extinction value of the absorption band.   *The intensity of the color decreases.*

(c) *Bathochrome.*   A shift of the absorption band towards the red (to
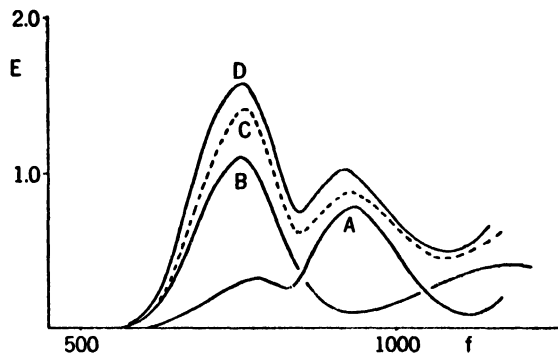
Fig. 2.—Separated resonators. A and B are absorption spectra of two monoazo dyes; C the addition of these curves; and D the absorption spectrum of a disazo dye containing resonators A and B coupled by an insulating link. (Brode, 4.)
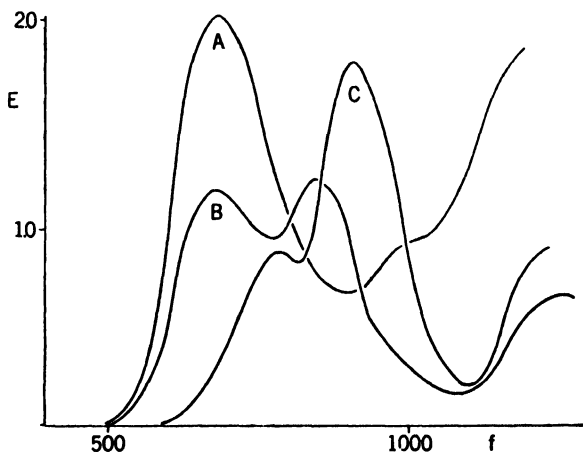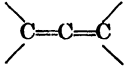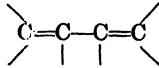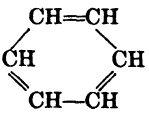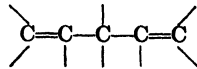
Fig. 3.—Conjugated and cumulative resonators. Absorption spectra of disazo dyes, A with the two resonators, B with the two resonators cumulatively linked and C with the two resonators separated. (Brode, 4.)

higher wave length or lower frequency values). *The color deepens* (*i. e.*, a change from yellow, to red, to purple, to blue, to green).

(*d*) *Hypsochrome.* A shift of the absorption band towards 'the blue (to lower wave length or higher frequency values). *The color lightens* (*i. e.*, a change from green, to blue, to purple, to red, to yellow).

Many of the simple resonators or chromophores do not in themselves produce visible absorption. Ethylene, for example, has an absorption band at 195 mμ in the extreme ultraviolet, but the conjugated coupling of two or more ethylene radicals together results in both hyperchromic and bathochromic effects. The actual effect seems to be more nearly a multiplication of resonance than addition. The carotenoid colors, such as those found in the carrot and tomato, are typical of a long chain of seven to thirteen conjugated double bonds.

TABLE II

CHROMOPHORE COMBINATION

Type	Formula	Examples
Cumulative	\C=C=C/	$CH_2=C=O$ Ketene
Conjugated	\C=C—C=C/	$CH_2=CH—CH=CH_2$ 1,3-Butadiene $CH_2=CH—C=O$ $\vert$ OH Acrylic acid CH=CH CH CH CH—CH Benzene
Separated	\C=C—C—C=C/	$CH_2=CH—CH_2—CH=CH_2$ 1,4-Pentadiene

The combination of resonators may be cumulated, conjugated, or separated (Figs. 2 and 3 and Table II). Cumulated resonators are those resonators which overlap or share points in common, usually resulting in a reduction in intensity and no marked change in wave length.

The conjugation of a number of resonators results in a bathochromic and

hyperchromic effect (Fig. 4). There seems to be, however, an upper limit to the length of a conjugated system; and in the absence of stabilizing end groups there is a decrease in molecular stability with an increase in the number of conjugated resonators.

In the conjugation of separate resonators to produce larger resonators a variation in the expected bathochromic effect results from the nature of the separate resonators and the end groups in the larger or composite reso-
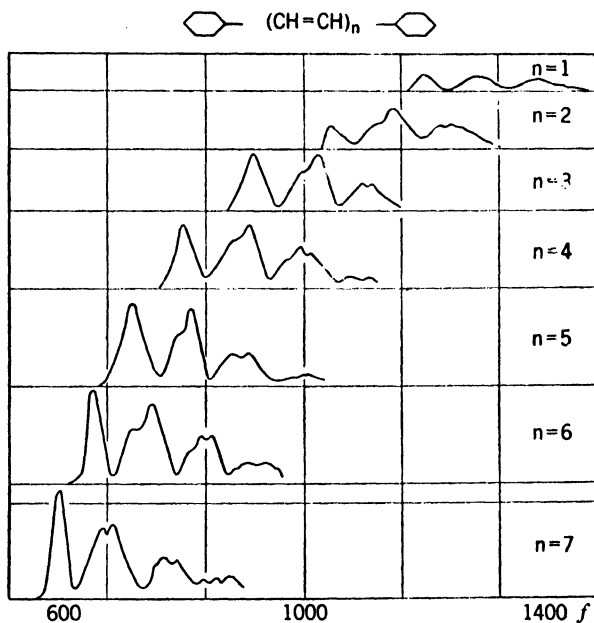
Fig. 4.—Effect of conjugation in polyethylene diphenyl derivatives C_6H_5—$(CH=CH)_n$—C_6H_5. (Kuhn *et al.*, 39.)

nator. Ethenylene linkages and phenylene linkages (—CH=CH— and —⟨ ⟩—) as the connecting resonator between end phenyl groups show a different degree of bathochromic effect (9).

Two types of conjugated resonators are recognized: (*a*) that in which there is a variation in the number of ethenylene or unsaturated linkages in the electromeric forms; and (*b*) that in which the resonating or electromeric forms have the same number of ethenylene or unsaturated linkages. Examples of these types are shown in the diphenylpolyene compounds (*a*), and the cyanine dyes (*b*).

(a) $\overset{..}{R}-\overset{|}{C}\!=\!\overset{..}{C}-\overset{..}{R} \ \underset{\longleftarrow}{\longrightarrow} \ R\!=\!\overset{|}{C}-\overset{|}{C}\!=\!R$

(b) $R\!=\!\overset{|}{C}-\overset{..}{R} \ \underset{\longleftarrow}{\longrightarrow} \ \overset{..}{R}-\overset{|}{C}\!=\!R$
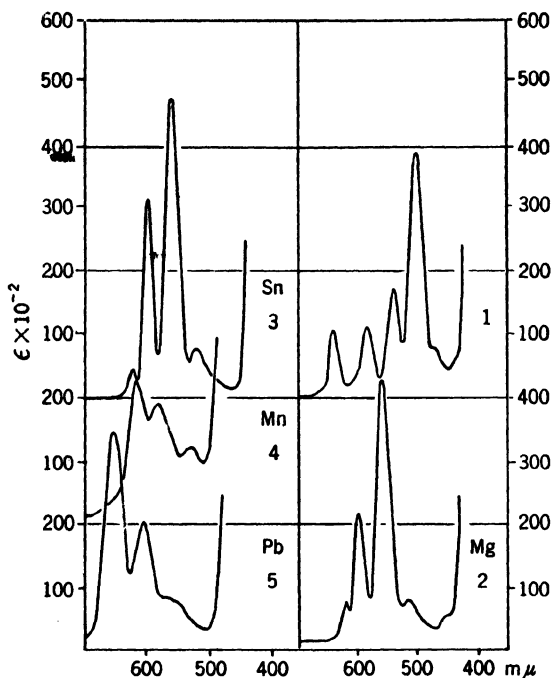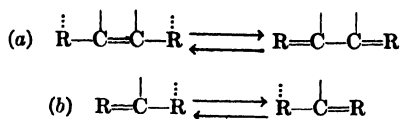
Fig. 5.—Influence of coordinated magnesium (2), tin (3), manganese (4), and lead (5) on the absorption spectrum of a synthetic porphyrin (1).   (Knorr and Albers, 37.)

   In addition to the combination of resonators and the presence of weighting groups, side chains and polar conditions will influence the position and intensity of the absorption band.   In general, an increase in mass results in a reduction in band frequency, although the position of the substituted group may result in a separation of resonators so as to produce a hypsochromic effect.

   The addition of weighting groups (37) usually produces a hyperchromic effect in the band of lower frequency and a hypsochromic effect in the bands of higher frequency (Fig. 5).

The change in electronic character of resonators (3) with a variation in pH or oxidation-reduction potential will produce absorption spectra changes which enable the identification or characterization of the resonator. Among these characterizing effects are isosbestic points (points of constant extinction) (Figs. 6 and 21, see page 303).

For further discussion of these relations between structure and absorption, the reader is referred to Brode (4), Morton (52), and Miller (48), and to reviews by Hogness and Potter (28), Loofbourow (43), and Heyroth (23). The rather brief outline of their effect as given in the preceding
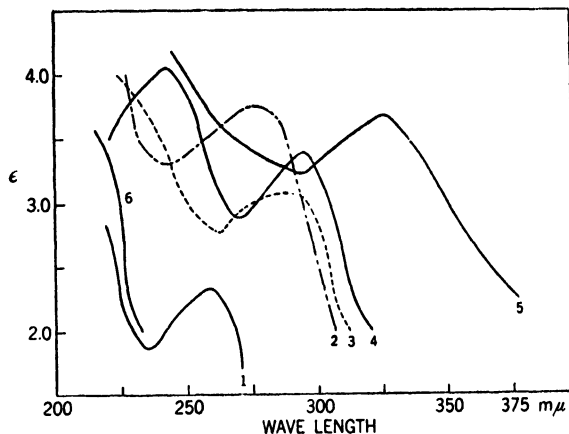
Fig. 6.—Absorption spectra of amino acids, (1) phenyl-alanine, (2) tryptophane, (3) diiodotyrosine, (4) tyrosine, (5) thyroxine, and (6) histidine. (Marenzi and Vilallanga, 46.)

portion of this chapter is intended to signify a few of the characteristics of a molecule that can be indicated through a study of its absorption spectra. Fortunately, many of the vitally important compounds such as vitamins, hormones, and enzymes have resonating properties and show selective absorption. The simple saturated fats, carbohydrates, and many proteins do not exhibit selective absorption, so that many natural compounds can be studied and measured spectrographically without actual separation from their solvent medium.

In the discussion of hormones, vitamins, and coenzymes in this and other volumes of this series, it is to be noted that a number of these substances have been isolated and their structure determined through a study

of their absorption spectra. In many of the chapters of this series will be found absorption spectra data and discussions of the application of the data to the determination of structure, as, for example, in *Advances in Enzymology*, Vol. III, pp. 6, 141, and 299.

III. Biologically Essential Compounds, Including Several Vitamins

Many naturally occurring compounds of physiological importance do not show any absorption within the usual ranges over which measurements are made, *i. e.*, 200 to 800 mμ. Among these are the saturated fats, the carbohydrates (excepting those in the free aldehyde form), and many of the simple proteins such as those containing alanine, serine, glycine, valine, etc. As has been pointed out in the preliminary discussion in this chapter, the essential prerequisite for absorption is a resonating structure; and this is usually characterized by unsaturation or double bonds in the structure. The transparency of completely saturated and nonresonating structures renders them very suitable as solvents for the study of resonating compounds. It also makes unnecessary the extraction or removal of such nonresonating compounds when observing the absorption of a resonating substance in their presence. Some of the vitamins and other essential biochemical materials are of a saturated, nonconjugated nature. As a result, they do not lend themselves readily to determination or analysis by absorption spectra unless they can be converted by chemical reaction to substances which do show resonance or can be coupled to some resonating structure in a quantitative reaction. Among the nonabsorbing, and hence nonresonating, essential compounds are biotin, pantothenic acid, unsaturated acids, choline, and certain amino acids.

1. Biotin

Biotin (vitamin H) has been shown to be similar to coenzyme R. It is obtained from liver extract and is a cyclic urea derivative with the composition $C_{10}H_{16}O_3N_2S$. Its structural formula has recently been shown to be a bicyclic compound yielding adipic acid on degradation and hence containing a 6-carbon continuous chain. It shows no characteristic absorption in either the visible or ultraviolet regions of the spectrum (64).

HN—CO—NH
HC————CH
H$_2$C—S—CH—CH$_2$—CH$_2$—CH$_2$—CH$_2$—COOH
Biotin
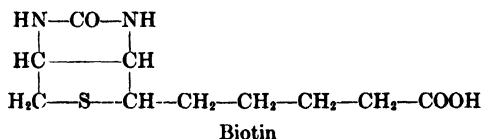
Derivatives of biotin have been prepared by condensation with phenanthraquinone to yield a quinoxaline. This reaction is characteristic of the combination of 1,2-diamines with o-quinones. The identity of the quinoxaline was confirmed through a study of its absorption spectra and a comparison with the absorption spectra of known quinoxalines (64).*

2. Pantothenic Acid

Pantothenic acid has been closely associated with the vitamins, and is considered an essential compound for the growth and nutrition of a number of plant and animal species, including man. It is an antidermatitis factor, and is essential for reproduction in chicks. Its formula has been determined as α,γ-dihydroxy-β,β-dimethylbutyryl-β-alanide.

$$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—COOH$$
Pantothenic acid

It has no unsaturation, other than in the carboxyl group, which does not in itself produce resonance or absorption within the observable region.

3. Choline

Choline is essential for fat metabolism and growth, and has sometimes been classed as a vitamin. It has no unsaturation and shows no selective spectral absorption. Choline is a quaternary ammonium base with the formula $(CH_3)_3N(OH)CH_2CH_2OH$.

4. Amino Acids

Certain of the amino acids have been found to be essential; their absence from an otherwise balanced diet will cause marked deficiency symptoms. The simple amino acids and their conjugated forms which produce proteins do not possess unsaturation other than the carboxyl and amide or peptide linkages which are not conjugated together and produce no selective absorption in the ultraviolet region. There are a number of amino acids (thyroxine, phenylalanine, tyrosine, etc.) which have resonating substituents attached like the radical (R) in the simple amino acid, R—CH-(NH_2)—COOH (13, 46) (Fig. 6).

In view of the fact that certain enzymes, coenzymes, and other important natural products have protein chains attached, it is significant to consider the absorption possibilities of the protein portion of the molecule (cf. later discussion on proteins in this chapter).

* See also Advances in Enzymology, Vol. III, p. 298.

5. Unsaturated Fatty Acids

There has been some tendency to class incorrectly as vitamins certain of the unsaturated fatty acids such as arachidonic, linolenic and linoleic acids, in view of their apparent essential character in the diet of rats to prevent dermatic lesions and necrosis of the tail. The determination of the degree of conjugation of fatty acids and the quantitative analysis of mixtures of unsaturated fatty acids have been in part solved through
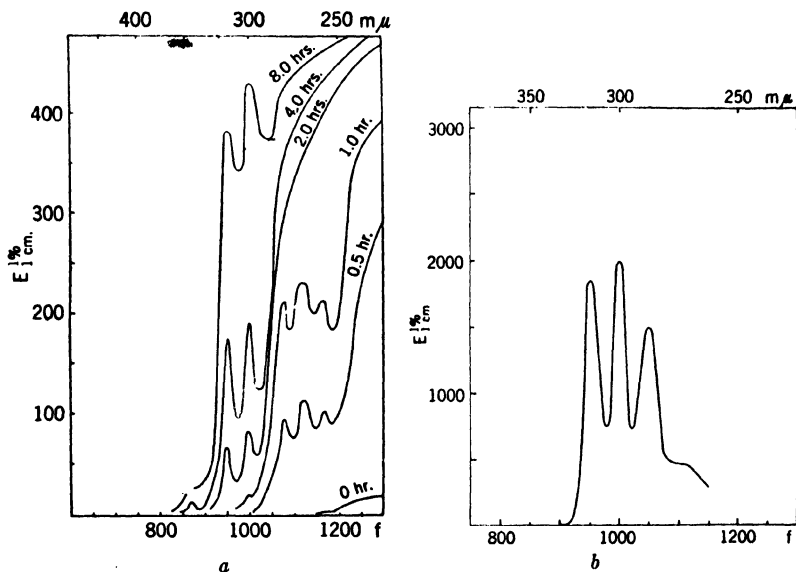
Fig. 7.—(a) Absorption spectra in isomerized unsaturated acids. Curves indicate absorption spectra of the unsaturated acid heated with alkali for the indicated time.
(b) Absorption spectrum of isomerized arachidonic acid with four conjugated double bonds. (Mowry *et al.*, 55.)

the use of absorption spectra methods (35, 50, 55, 57). The fact that normal, nonconjugated polyeneic fatty acids can be converted to conjugated acids enables one to determine the number of conjugated linkages in the original molecule from the extent to which conjugation can be induced. With each additional double bond which is conjugated within the resonating structure there is a bathochromic and hyperchromic shift in the absorption spectrum. Since, for the molecules containing a limited number of conjugated linkages, the position and character of these absorption bands

are well known and defined (Fig. 7), it is possible to indicate with certainty the number and approximate location of the unsaturated linkages from a study of the absorption spectrum and the rate of conjugation (Fig. 7a). The structure of arachidonic acid has thus been definitely determined through a study of the absorption spectra and degradation products (55). In mixtures containing oleic, linoleic, linolenic, and arachidonic acids or their glycerol esters, it has been possible to determine the amount of each of these through a study of their absorption spectra (57).

6. Penicillin

Recent studies on penicillin have indicated a decrease in spectral absorption of penicillin extracts with an increase in their purity and an inactivation of penicillin extracts without causing a change in absorption. This would mean that both penicillin and its decomposition products do not exhibit selective absorption (30).

7. Vitamin A and Carotenoids

Vitamin A ($C_{20}H_{29}OH$) is a conjugated unsaturated alcohol derived from certain provitamins known as carotenoids. Among the number of carotenoids which are isomeric in character and are closely related to vitamin A are α-, β-, and γ-carotenes and lycopene, all of which have the same empirical formula ($C_{40}H_{56}$). They are brilliant in color and, as would be predicted of highly unsaturated hydrocarbons, are fat-soluble. Their color is due to the highly conjugated structure shown in the accompanying formula:
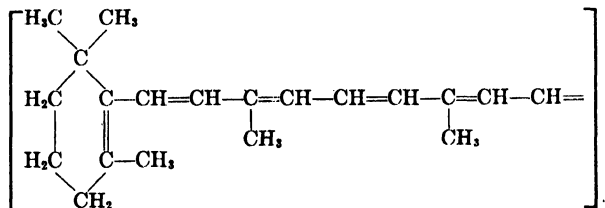
Conjugated Structure of Isomeric Carotenoids of Formula, $C_{40}H_{56}$

The slight variation in the number and arrangment of the conjugated double bands in carotenoids results in changes in the absorption spectra curves, thus enabling one to identify these compounds, for example, the isomeric hydrocarbon carotenoids, α-, β-, and γ-carotenes (Fig. 8). Oxidation, saturation, and cyclization of the carotenes to form alcohols,

TABLE III

RELATION OF ABSORPTION SPECTRA TO NUMBER OF CONJUGATED BONDS

Compound	Double bonds, total	Double bonds, conjugated	Double bonds, nonconjugated	Wave length, mμ		
				SbCl₃	CS₂	Hexane
α-Carotene	11	10	1	589	511	478
β-Carotene	11	11	0	590	520	485
γ-Carotene	12	11	1	590	533	494
Lycopene	13	11	2	585	546	506

ketones, and less highly saturated derivatives produce a marked change in the absorption spectra. Since these carotenoids are soluble in fats, which in themselves are optically transparent in the ultraviolet, it becomes
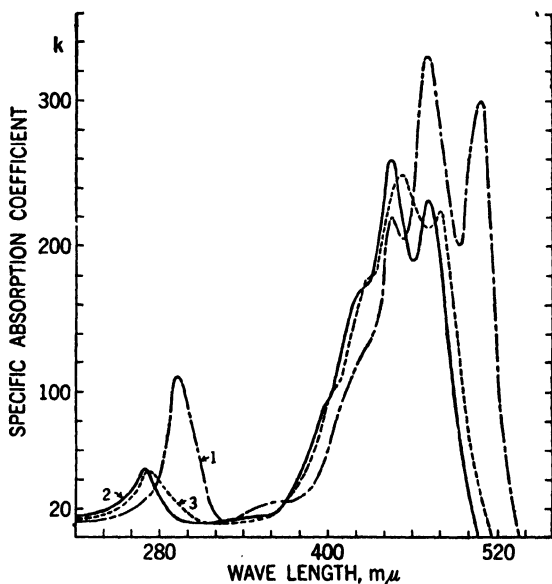
Fig. 8.—The absorption spectra of (1) lycopene, (2) α-carotene, and (3) β-carotene. (Miller, 48.)

possible to identify them and estimate their concentration in many cases without actually isolating the compounds (53).

The reaction of antimony trichloride in chloroform solution (5, 7, 54) with certain types of unsaturated compounds produces a brilliantly visible, colored solution for which sharp absorption bands are characteristic of

the unsaturated compound. The test with antimony trichloride has been used extensively in the colorimetric estimation of the vitamin A content of fish oils.

Vitamin A, which is produced *in vivo* by a fission and hydrolysis of the carotene nucleus, has the structure given in the accompanying formula.

$$H_3C \quad CH_3$$
$$\diagdown \diagup$$
$$C$$
$$\diagup \diagdown$$
$$H_2C \quad C—CH=CH—C=CH—CH=CH—C=CH—CH_2OH$$
$$H_2C \quad C—CH_3 \quad CH_3 \quad CH_3$$
$$\diagdown \diagup$$
$$CH_2$$
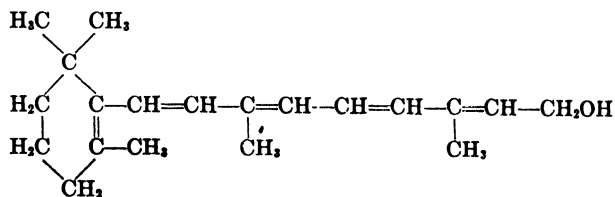
Vitamin A

Since it has only five conjugated double bonds, it shows selective absorption in the near ultraviolet (325 mμ) rather than in the visible region of the spectrum (Fig. 9).
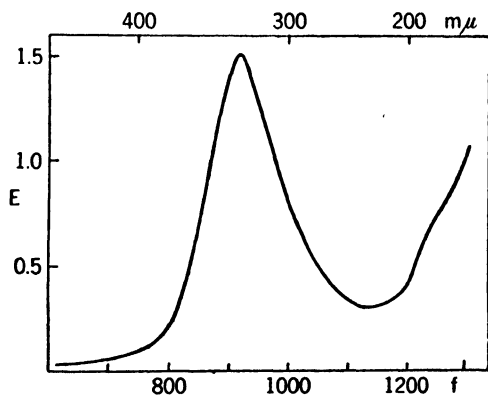
Fig. 9.—Absorption spectra of vitamin A containing fish oil.

In the earliest work on carotene, only one form of the compound was believed to exist and one unit value of potency was assigned to it. However, with the discovery and characterization of the α, β, and γ forms, the unit value for carotene has lost significance. An examination of materials containing vitamin A has shown that in addition to the normal absorption band at 325 mμ and the antimony trichloride band at 617 mμ, certain sources of vitamin A, and in particular the halibut-liver oils as compared with the cod-liver oils, give maxima at 340 and 622 mμ in the

normal and antimony trichloride solutions (52). The potency of these halibut-liver oils is such as to require a factor in the determination of the vitamin potency different from the extinction values. It would thus appear that there must be more than one vitamin A. The designation of vitamin A_2 has been assigned to the form with a slightly greater wave length of absorption. The increased wave length would indicate a longer resonator or an increase in the weighting of the resonator. It is quite possible that there may be a number of compounds with a varying degree of potency and possessing somewhat similar structures, but with sufficient difference so as to be distinguishable by their absorption spectra.
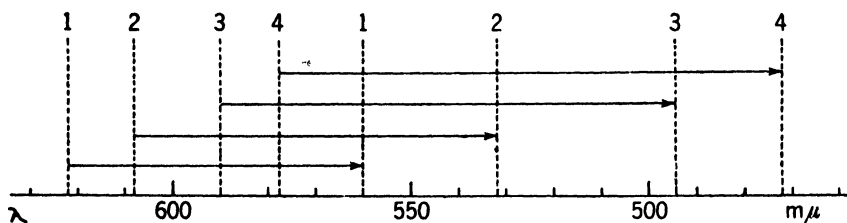
Fig. 10.—Shift of absorption bands centers as a result of fading of antimony trichloride color reaction compounds produced with vitamins: 1, 2, 3, and 4 are band centers of fresh solutions and 1′, 2′, 3′, and 4′ are band centers which develop on fading of the original color. (Brode and Magill, 6.)

As a further evidence of the existence of similar resonators of different size or weighting it should be noted that the antimony trichloride bands of these different compounds—vitamin A, vitamin A_2, β-carotene, etc.— all fade to give bands which are in the center of the visual spectrum but still maintain a proportional difference in wave length (Fig. 10).

The absence of absorbing materials other than vitamin A in many oils has led to the use of the extinction at 325 mμ as a satisfactory and rapid method for the estimation of vitamin content in many natural oils. As has been indicated above, there are some oils which do not conform, but for many types the method has been accepted as a standard method of analysis.

The generally accepted data for vitamin A would indicate an extinction, E (325 mμ), of 1880 $\pm$40 for the ultraviolet absorption and an E (617 mμ) of 6000 $\pm$200 for the antimony trichloride reaction color. The potency based on rat tests as compared with pure β-carotene has been given as 3.0 to 3.3 $\times$ 10^{-6} I.U. per gram. A conversion factor can thus be set up of

1600 to 1750 for the direct conversion of $E_{1cm.}^{1\%}$ 325 mμ to I.U., *i. e.*, $E_{1cm.}^{1\%}$ 325 mμ × 1600 = I.U. (This factor is subject to some revision.)

Morton (52) has indicated possible procedures for the estimation of relative concentrations of A and A$_2$ through a comparison of their antimony trichloride spectral intensities at 620 and 695 mμ. A suggested formula for A$_2$ has indicated the existence of one additional ethenylene linkage in the unsaturated chain.

8. *Inositol and p-Aminobenzoic Acid*

Inositol as a saturated cyclic hexahydroxy alcohol exhibits no absorption. This compound has been associated with the "B family" of vitamins.

Another of the associated compounds in the "B family," *p*-aminobenzoic acid, shows a distinct and characteristic absorption, and in addition gives, through its ability to be diazotized and coupled, additional resonating compounds which permit its exact estimation and identification.

IV. Hormones

The classification of the hormones is not so well defined as the classification of vitamins. In many cases there appears to be an overlapping or close relationship between certain vitamins and hormones.
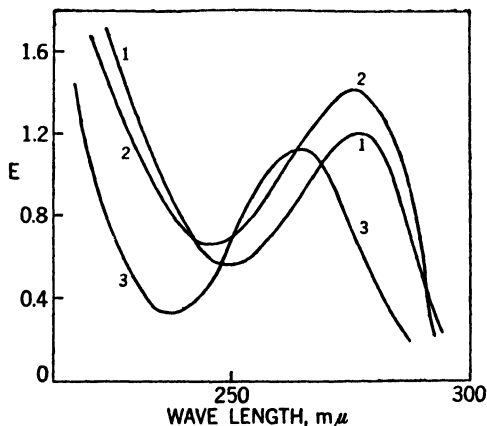
Fig. 11.—Absorption spectra of (1) Adrenaline: (HO)$_2$C$_6$H$_3$·CH(OH)·CH$_2$·NHCH$_3$. (2) Catechol: (HO)$_2$C$_6$H$_4$. (3) Phenylalanine: C$_6$H$_5$CH$_2$·CH(NH$_2$)·COOH. (Lopez, 45).

1. Adrenaline

Adrenaline is sometimes considered as a hormone. In view of its aromatic character, it exhibits a definite absorption spectrum (Fig. 11). However, since its absorption is due to a resonating benzene ring with a nonresonating substituent attached, it is obvious that its spectrum will not differ markedly from other similar but nonhormone active compounds (13, 45) from the physiological point of view.

2. Thyroxine

The thyroid hormone, while not identified as the free amino acid thyroxine, is closely related to this compound. It is also felt that the hormone may not be identical with thyroglobulin (21). Thyroglobulin has, however, been shown to be the storage form of this hormone. As such, its analysis and identification must be closely allied with the determination of the hormone itself.

Thyroglobulin consists of a protein–thyroxine complex. Since thyroxine

$$HO \overset{I}{\underset{I}{\diamondsuit}} O \overset{I}{\underset{I}{\diamondsuit}} CH_2\!-\!CH(NH_2)\!-\!COOH$$
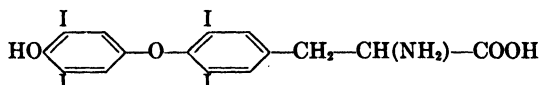
Thyroxine

has an aromatic structure, it shows a characteristic absorption which can be used to identify it and aid in its estimation. Recent work (56) has indicated that thyroxine possesses a quinoidal activity, the ultimate proof of which must be based largely on absorption spectra data.

3. Insulin

Insulin does not show in its near infrared absorption any difference in selective absorption from that shown by other proteins (2). Its ultraviolet absorption exhibits a band between 250 to 290 mμ, maxima at 275 mμ, with a specific extinction of 1015. This band has been suggested as a means of quantitative estimation of the compound. There are a number of other proteinlike substances showing absorption in this region, and hence the method must of necessity be limited to a concentration determination of material of known purity (59). The absorption band of insulin is associated with the cystine and the resin contents. Insulin can be inactivated without change of its absorption spectra; but any treatment which alters its absorption spectra results in inactivation (39).

4. Sex Hormones

Many of the known hormones, such as the adrenocorticotrophic hormone (ACTH), the gonadotrophic hormones (ICSH), (FSH), and (PU), the growth and metabolic hormones, and the hormones of the neurohypophysis, are known to be of a protein character and of very large molecular weight (21). It would therefore be expected that their absorption spectra would be similar to those of proteins, and as such would not be typical of the structure or specific properties of the substance. As has been pointed out, many of the simple amino acids do not exhibit selective absorption above 200 mμ, while others containing aromatic or other resonating groups do show a certain type of absorption (Fig. 6). In mixtures involving a large number of different amino acids in combination, it would be expected that the summation of the absorbing constituents would yield a more or less general absorption which would not be useful in identification or estimation.

5. Plant Hormones

3-Indoleacetic acid shows the characteristic absorption of the indoles. As would be expected from the known insulating effects of the methylene and ethylene linkages, there is no apparent difference between the absorption spectra of 3-indoleacetic acid and 3-indolepropionic acid, although the former is very active as a plant-rooting hormone and the latter is practically inactive. The absorption here is due to the indole structure

TABLE IV
COMPARISON OF TWO PLANT HORMONES (52)

Factor compared	3-Indoleacetic acid		3-Indolepropionic acid	
Hormone property	Active		Inactive	
Absorption maxima, mμ	280	225	281	226
Log ϵ	3.8	4.5	4.0	4.6

which, while a part of the essential hormone structure, is not in itself of a hormone character; the additional structure necessary for hormone activity does not influence the absorption or resonance of the indole, although it is essential for the hormone activity. 1-Naphthaleneacetic acid is another similar compound which shows absorption, due to the naphthalene structure but requires the acid radical substituent in order to possess an hormone root-forming activity.

6. Steroid Hormones

Contrasted with the nonselective absorption of the previous types of hormones, the steroid hormones possess a definite and characteristic absorption (Fig. 12) which is due to the resonating aromatic character of compounds. In the discussion of vitamin D, reference is made to the steroid hormones which are closely associated with the steroid vitamins.
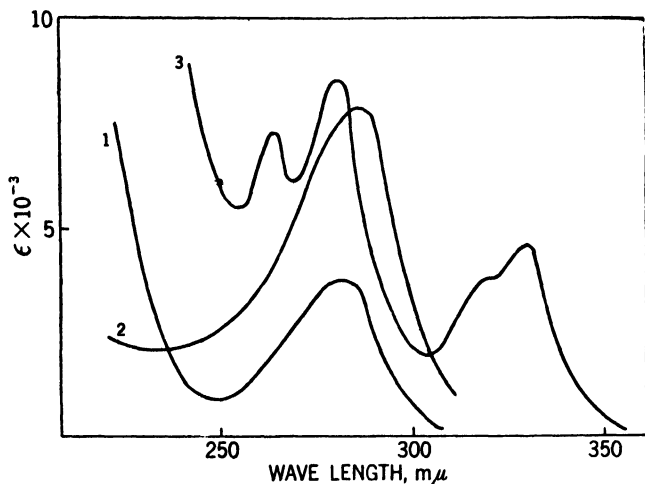
Fig. 12.—Absorption spectra of steroids: (1) estrone (the curves for equilin and estriol are identical with the curve for estrone); (2) androsterone; and (3) equilenin. (Dirscherl and Hanusch, 11.)

Among the steroid hormones which have been isolated and structurally identified are such compounds as equilin, estrone, and androsterone. These sex hormones usually have an aromatic phenolic ring like the "A" ring of the sterol structure, and in many cases possess a quinoid structure which gives enhanced absorption (Fig. 12) (52). The similarity of absorption of equilin and estrone indicates that the additional double bond in equilin is not conjugated with the phenol ring. The absorption spectra produced by the dehydrogenation of equilin to produce equilenin indicate a naphthol structure which is confirmed by comparison of the absorption spectra of simple phenol (Fig. 1) and naphthol derivatives.

Estrone and equilin have a carbonyl group in position 17 and yet show as their principal absorption the curve which is characteristic of phenol,

STEROID HORMONES

Estrone   Estriol   Progesterone

Androsterone   Equilin   Equilenin
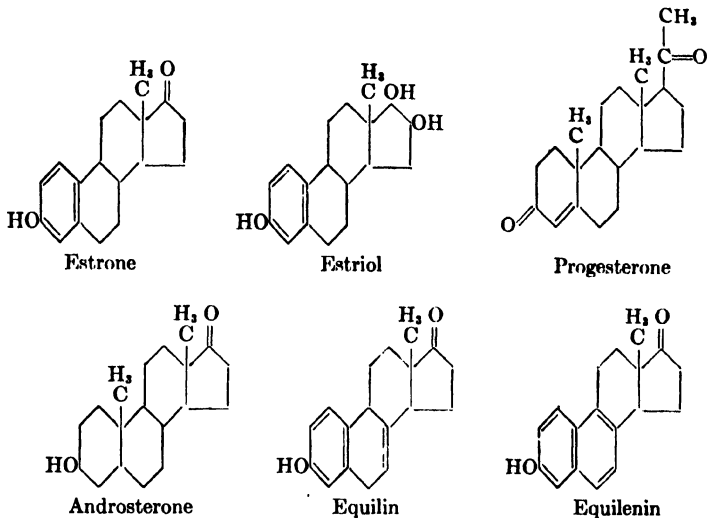
which is the stronger resonator. It thus appears that the separation of the "A" ring in steroids from the carbinol, carbonyl, or radical substituent at position 17 by an insulating connecting chain prevents the transmission of effects which are sufficient to produce noticeable changes in the absorption spectra of the "A" resonator; hence it is not possible to use the absorption method of investigation to determine the character of the substituents at position 17 (cf. formulas given in the discussion on vitamin D for the significance of the numbered ring positions).

In some steroid hormones, such as androsterone, testosterone and progesterone, the "A" ring appears as an α-β unsaturated ketone with entirely different spectral characteristics from the phenolic type of absorption.

The similarity in structure and absorption spectra of many steroid hormones limits to some extent the usefulness of absorption spectra methods in identification and estimation. However, the absorption spectra of pure compounds have been of considerable aid in assigning the location of double bonds in the partially saturated structures.

V. Vitamin D

The steroids involve a large group of alcohols, acids, and related substances which occur in many important natural products, including

certain vitamins and hormones.   These compounds are high molecular weight cyclic substances which involve a fused ring structure such as:

CH_3
H_3CH—CH_2—CH_2—CH_2—CH—CH_3

*Side chain at 17 numbered from 20 upwards
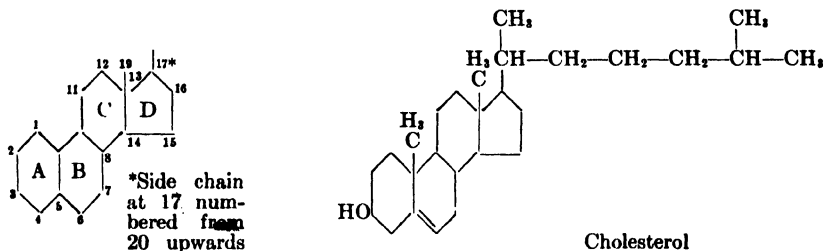
Cholesterol

The ring system may be saturated or unsaturated, and may have certain ring cleavages.   Saturated hydrocarbons such as androstane and alcohols such as cholesterol exhibit no selective absorption.   Unsaturated compounds derived from the parent saturated compounds show marked absorption and, as has been indicated in the preceding general discussion on the theory of absorption spectra, the conjugation of these unsaturated linkages results in marked bathochromic and hyperchromic effects (Fig. 13) (27).
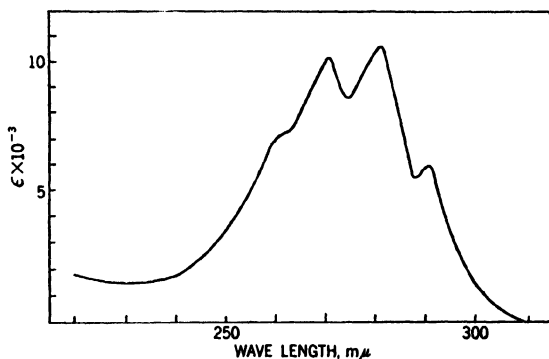
Fig. 13.—Absorption of 7-dehydrocholesterol (curve is identical with that of ergosterol).   (Hogness, 27.)

In the studies on cholesterol it was found that the highly purified material did not possess antirachitic property nor did it have a definite absorption spectrum.   Impure cholesterol, however, did show antirachitic properties and selective absorption.   Concentration of this absorbing material into a less soluble portion resulted in the isolation of a highly

potent fraction. The similarity of the absorption curves of ergosterol, calciferol, and vitamin D (52) would indicate that, insofar as the resonating portions of the molecules were concerned, a similar structure existed.

Ergosterol (provitamin D₂)   Vitamin D₂ (calciferol)
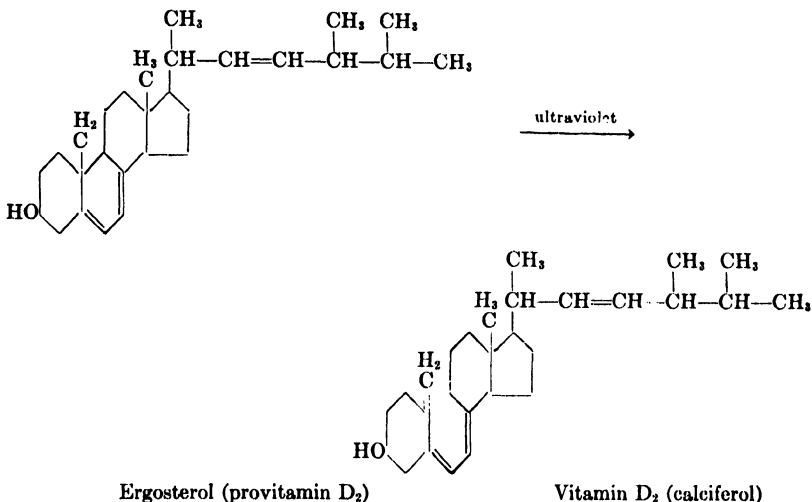
Vitamin D₃ is similar to vitamin D₂, except that the
double bond at 22–23 is saturated with hydrogen.

It should be noted that similar structures give similar absorption; and yet the slight difference in structure may be sufficient to give markedly different physiological properties, evidenced by the nonantirachitic character of ergosterol as compared with the antirachitic properties of vitamin D and calciferol.

There are a number of steroid compounds with similar absorption spectra. Concomitantly one would expect to, and does, find that there are several similarly constituted compounds with strong antirachitic property. The antirachitic steroids show absorption spectra typical of conjugated unsaturation. The fact that calciferol has one more double bond than vitamin D is not indicated in a comparison of their absorption spectra since the extra double bond in calciferol is located in the 22–23 position and is not conjugated with other unsaturated linkages.

Irradiation with ultraviolet light may cause isomerization with a resulting conjugation change, and hence a change in the absorption spectra. In the case of ergosterol, irradiation also produces calciferol, with an antirachitic property the potency of which is concomitant with an absorption spectra change.

The direct estimation of vitamin D by spectrographic methods is limited in application because its absorption band at 265 mμ is masked by sterol absorption in the 325 mμ band of vitamin A, which often occurs along with vitamin D in unsaponifiable oil fractions. The higher molecular extinction of vitamin A and the longer wave length position of its band are such that in an oil containing 0.5% vitamin A the absorption band of 10% vitamin D in the same oil will be masked.

The work on vitamin D has shown that there are several compounds which possess antirachitic properties and each of which has a different provitamin, ergosterol being the provitamin for vitamin D_2 and 7-dehydrocholesterol for vitamin D_3. There are considerably greater differences between the observed potencies of these different vitamin D materials than between their absorption spectra. This same variation in the relation of potency to absorption is also evident for other natural products, e. g., the various vitamin A compounds.

Vitamin D gives a color reaction with antimony trichloride which shows a maxima at 500 mμ. The potency can be determined from the extinction of this colored solution on the basis of the formula $E_{1cm.}^{1\%} \times 19,300 =$ potency in U. S. P. units, where 19,300 is the conversion factor as determined from comparison with known standards (16). In the application of this method, it is essential to separate vitamin A from vitamin D by an absorption process, prior to the treatment with the antimony trichloride, since vitamin A also gives a marked color reaction with this reagent. It has been shown, however, that the presence of added amounts of ergosterol and cholesterol does not cause an appreciable change in the observed vitamin D content.

VI. Vitamin E

The tocopherols are aromatic derivatives of a chroman type, with an aliphatic side chain ($C_{16}H_{33}$) of a phytyl character and hydroxyl and methyl substituents on the aromatic ring. The number and position of the methyl groups on the aromatic ring differentiate α- from β-tocopherol.
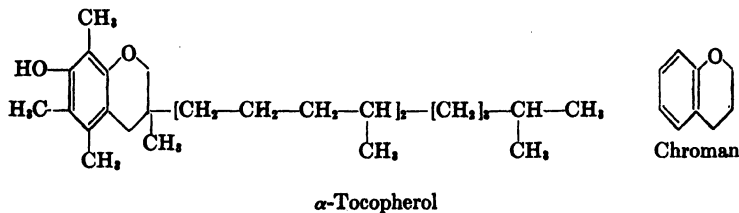
α-Tocopherol

With an hydroxyl group para to the chroman oxygen it would be expected that the tocopherols should show an absorption typical of mono-*o*-alkylated hydroquinone with some modification due to the chroman ring (Fig. 14) (60, 68).

The tocopherols have absorption maxima at about 294 mμ with $E_{1cm.}^{1\%}$ = 100. The concentration of tocopherol in wheat germ oil is so low that the extinction for an oil containing 0.5% should be about $E_{1cm.}^{1\%}$ 294 mμ = 0.5. Actually, an extinction value of about 2.0 is obtained, indicating
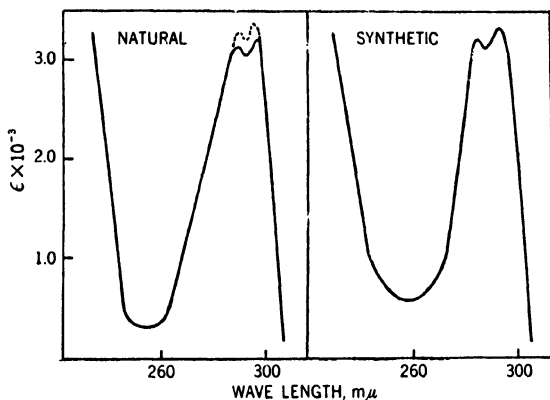
Fig. 14.—Absorption spectra of natural α-tocopherol and synthetic *dl*-α-tocopherol in hexane. (Smith, 60, Webb, 68.)

that other substances such as steroids constitute the major absorbing material and thus preclude the use of the absorption spectra method for the direct determination of vitamin E. Chromatographic adsorption can be used to separate the tocopherols from other absorbing substances, and fractions rich in vitamin E may respond to direct spectrographic analysis if their absorption due to the tocopherols is considerably greater than other agents absorbing at a wave length near 290 mμ.

VII. Vitamin K

The K vitamins (antihemorrhagic) vitamin K_1 and vitamin K_2, have been characterized spectroscopically by Karrer, *et al.* (32), who showed that the material obtained by concentration gave absorption bands at 248, 261, 270 and 328 mμ. The 328 mμ maximum was considerably lower than the others and disappeared on hydrogenation. Doisy and co-

workers (12) and Fieser and coworkers (19) published papers nearly simultaneously describing the final assignment of structures to vitamins K_1 and K_2, the former being obtained from vegetable sources and the latter from animal sources.
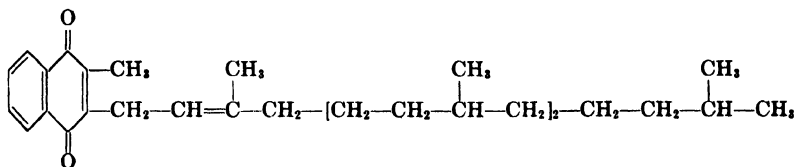
Vitamin K_1
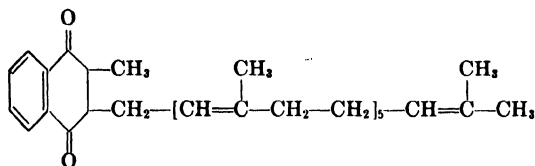
Vitamin K_2
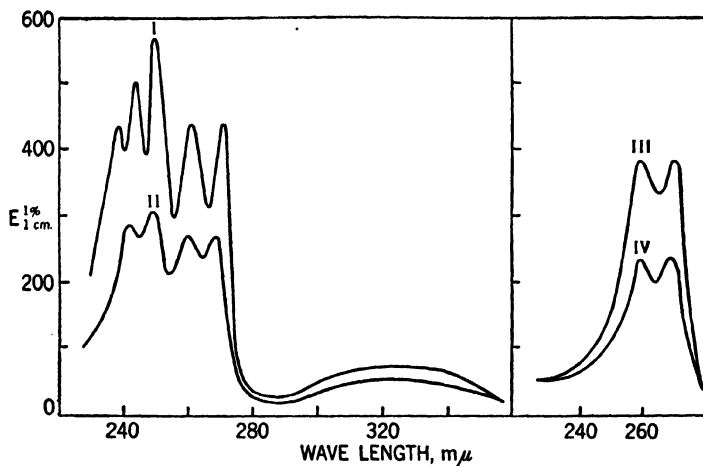
Fig. 15.—Absorption spectra of (I) vitamin K_1 (II), K_2 and their reduction products, (III) and (IV), respectively, tetrahydro derivatives. (Ewing *et al.*, 18.)

The discovery and isolation of vitamins K_1 and K_2 and the subsequent determination of their structure were guided to a considerable extent

through a study of their absorption spectra. These vitamins have as their essential absorbing or resonating structure the 1,4-naphthoquinone nucleus—the structure assigned to K_1 being 2-methyl-3-phytyl-1,4-naphthoquinone, and to K_2, being 2-methyl-3-difarnesyl-1,4-naphthoquinone. The similarity of the specific extinction curves of K_1 and K_2 would indicate that the resonating structures are alike, and that K_2 has a higher molecular weight, caused by a nonresonating attachment to the resonator which is attached at a position in the molecule not materially affecting the resonating molecule (Fig. 15).
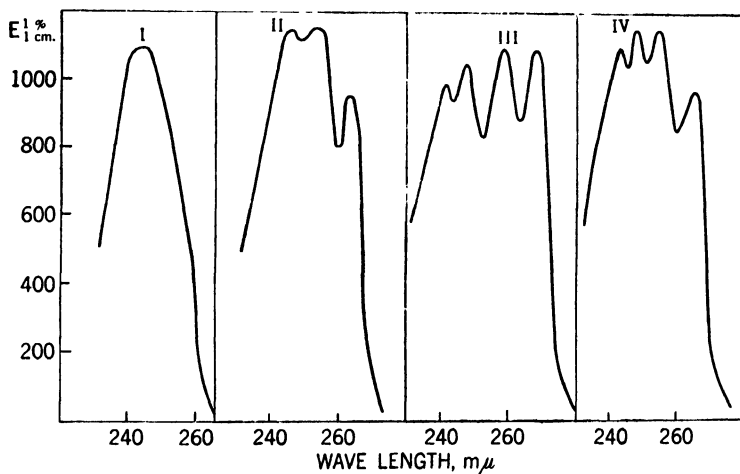
Fig. 16.—Absorption spectra of compounds related to vitamin K: I, 1,4-naphthoquinone;   II, 2-methyl-1,4-naphthoquinone;   III, 2,3-dimethyl-1,4-naphthoquinone;   IV, 2-ethyl-1,4-naphthoquinone. (Ewing et al., 18.)

The reduction of K_1 and K_2 leads to the formation of tetrahydronaphthoquinones with similar differences between their specific extinctions.

From Figure 16 it will be noted that the introduction of substituents in the quinone side of the naphthoquinone structure results in a marked variation in the observed absorption spectrum, although variation in the size of the group attached in a given position produces little if any effect on the observed absorption spectrum (18).

The effect of irradiation of vitamin K_1 with ultraviolet light is shown in Figure 17, in which it will be noted that there is a relation between the concentration of faded material and vitamin K_1, resulting in definite

isosbestic points at 230, 277, and 395 mμ.   These isosbestic points are also noted in resonator changes produced by pH or oxidation-reduction changes where both products in the reaction are resonating in character (17).
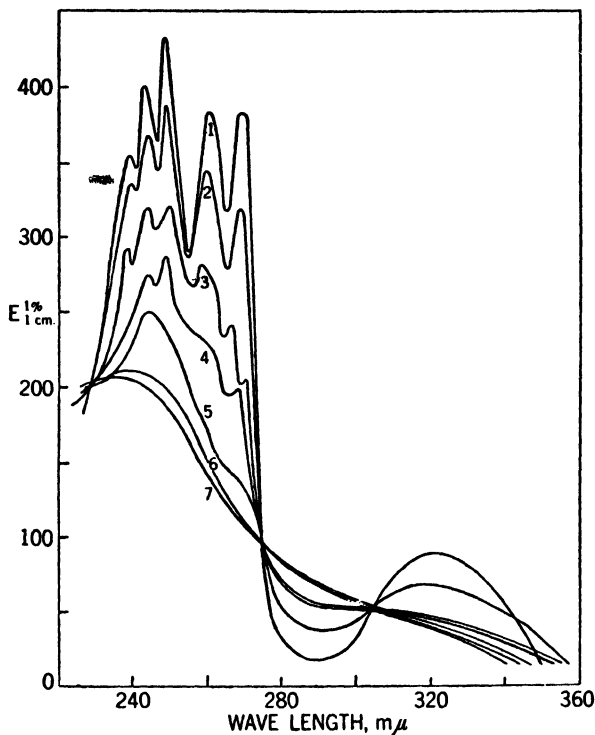
Fig. 17.—Effect of ultraviolet radiation on vitamin K. Time of radiation 0, 30, 60, 90, 135, 195, and 255 minutes for samples 1 to 7, respectively.   (Ewing *et al.*, 17.)

VIII.   Vitamin C

Vitamin C is an antiscorbutic vitamin whose structure has been definitely established and to which the name ascorbic acid has been given.   The vitamin is an unsaturated hydroxy acid with the unsaturated linkage conjugated to the carboxyl group.   Studies on simple unsaturated acids with the ethenylene linkage conjugated with and separated from the carboxyl have shown that the conjugation produces a bathochromic

and hyperchromic effect; hence it would be expected that ascorbic acid would show definite absorption.

$$CH_2OH-CHOH-HC-C(OH)=C(OH)-CO$$
$$\underset{O}{\lfloor\qquad\qquad\qquad\rfloor}$$
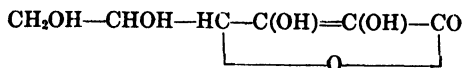
Vitamin C (*l*-ascorbic acid)

The vitamin is easily oxidized, an action catalyzed by ultraviolet light. It is therefore essential that the solvent for the absorption spectra determination be free from oxygen and that the determination be made as rapidly as possible. The oxidized form of ascorbic acid (dehydroascorbic acid) no longer contains an unsaturated linkage conjugated to the carboxyl and the compound exhibits no absorption in the usual ultraviolet range of observation.
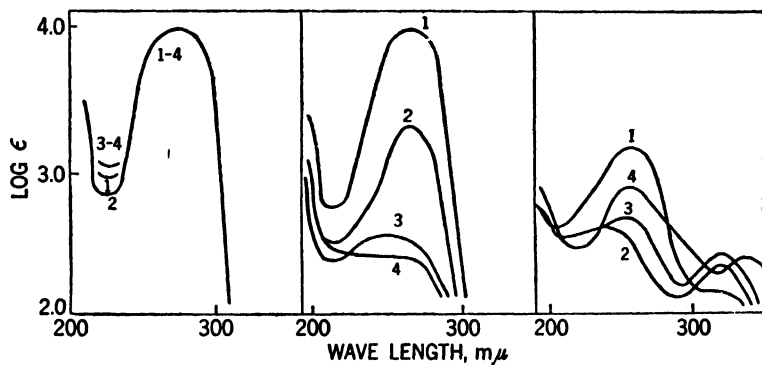
Fig. 18.—Effect on its absorption spectra of air bubbled through a solution of vitamin C: (1) fresh, (2) 1 hour, (3) 5 hours, (4) 24 hours. All solutions contain $M/2000$ ascorbic acid. *Left*, solution stabilized with $M/2000$ KCN; *center*, untreated solution; and *right*, oxidation accelerated by $M/20,000$ CuSO$_4$. (Mohler and Lohr, 51.)

The change in absorption with oxidation has provided a convenient method for studying the rate of oxidation, its catalysis, and methods of preventing oxidation in dilute solutions. Mohler and Lohr (51) have shown that copper salts catalyze the oxidation process and that potassium cyanide acts as a preservative in preventing the oxidation process (Fig. 18).

This oxidation process has been used to determine the amount of ascorbic acid through a comparison of the absorption spectra of samples before and after treatment with a dilute copper solution and time allowed for the

oxidation process. The difference in extinction of the two samples indicates the amount of the vitamin in the material (31).

The absorption maximum of ascorbic acid varies, with changes in concentration and solvent, from 245 to 265 mμ, although its extinction value of log ϵ 3.97 remains fairly constant. This extinction is considerably higher than for analogous compounds such as acetylpyruvic acid and dihydroxymaleic acid, and may be due in part to the ring strain in the lactone bridge structure of the ascorbic acid.

Two recent methods (25, 38) have been proposed for the estimation of ascorbic acid through the observed extinction values of compounds produced by the interaction of the vitamin with certain reagents. In one of these (38), ferripyridyl is combined in excess with the vitamin, and is reduced by it to ferropyridyl, which is a stable red ion whose concentration, as observed spectrophotometrically at its absorption maximum of 510 mμ, is proportional to the amount of ascorbic acid. The second method (25) is based on the rate of decoloration of the dye, 2,6-dichlorophenolindophenol, under controlled temperature and pH conditions, the color intensity being determined spectrophotometrically from the absorption values at 520 mμ.

IX. Vitamin B

The large number of B vitamins which have been isolated and structurally identified differ so widely in their structure that they require a separate discussion of each classification.

1. Vitamin B_1

Vitamin B_1 is the antiberiberi and antipolyneuritis vitamin, and has been identified as thiamine hydrochloride. Its structure has been shown to be that of a thiazole pyrimidine; and while the thiazole and pyrimidine are not coupled through a conjugated linkage, each of these two units is a resonator and shows a definite and characteristic spectral absorption.
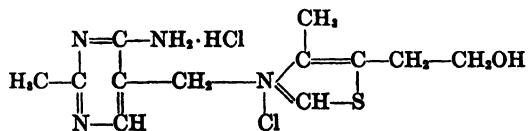
Vitamin B_1 (thiamine)

The identification and determination of the structure of vitamin B_1 is an excellent example of the application of absorption spectra methods to the identification of natural products with resonating structures. The natural vitamin B_1 had an absorption spectra as indicated in Figure 19a. After

cleavage with sodium sulfite, the molecule yielded an acidic and basic fragment (69):

$$C_{12}H_{16}N_4OS \cdot HCl + Na_2SO_3 \longrightarrow C_6H_9N_3OS_3 + C_6H_9NOS + 2NaCl$$

<div align="center">
Acidic   Basic
fragment   fragment
</div>
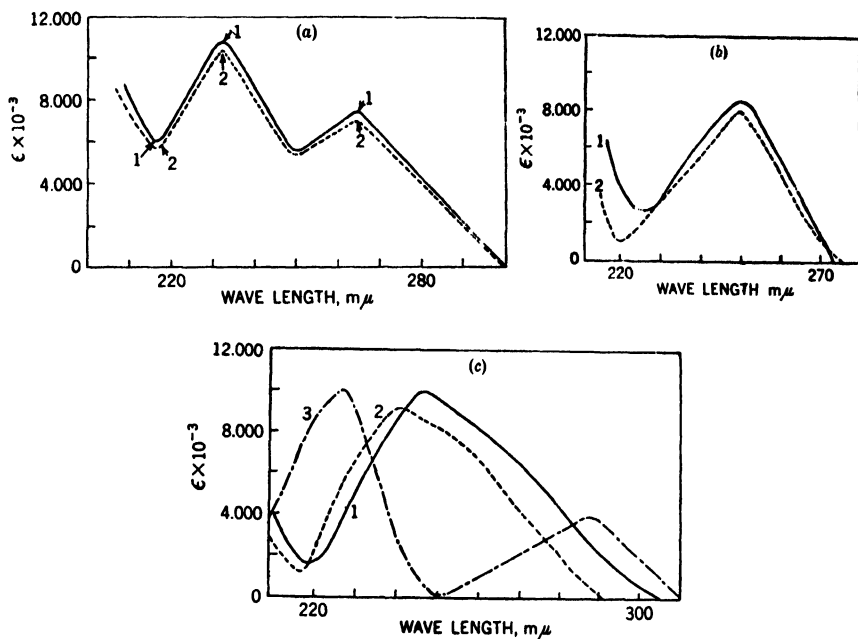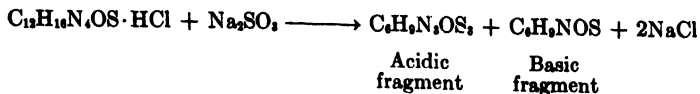
Fig. 19.—The absorption spectra of natural and synthetic vitamin B₁ and its cleavage product; (a)—(1) natural vitamin B_1, (2) synthetic vitamin B_1; (b)—(1) basic cleavage product, hydrochloride of vitamin B_1; (2) 4-methylthiazole hydrochloride; (c)—(1) amino sulfonic acid cleavage product of vitamin B_1; (2) 2,4-dimethyl-6-aminopyridine, (3), 4,6-dimethyl-2-aminopyridine. (Williams and Ruehle, 69.)

The basic fragment, on treatment with nitric acid, yielded a thiazolecarboxylic acid. From its known structure it was determined that the basic cleavage fragment was 4-methyl-5-(2-hydroxyethyl)thiazole. The marked similarity of the absorption spectra of the basic cleavage product,
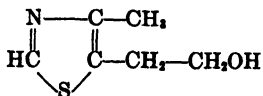
4-Methyl-5-(2-hydroxyethyl)-thiazole

as the hydrochloride and methiodide, as compared with the corresponding absorption spectra of the hydrochloride and methiodide of 4-methylthiazole (Fig. 19b) pointed to the probable structure of the cleavage product. The synthesized 4-methyl-5-carboxythiazole proved to have identical properties and absorption spectra with the oxidized basic cleavage product.

The acidic fraction proved to be a pyrimidine which also had a characteristic absorption spectrum. The pyrimidines are also found in nucleic acids and other important physiologically active compounds and have been rather carefully studied with regard to the influence of position of substitution on their absorption spectra. Treatment of the acidic cleavage product of vitamin B and sodium sulfite with sodium in ammonia resulted in regeneration of sodium sulfite and the formation of a pyrimidine which was subsequently identified as 2,5-dimethyl-4-aminopyrimidine. This latter compound, while giving pyrimidine reactions, did not conform in its absorption spectra to that of known pyrimidine derivatives. Hence it was necessary to synthesize new pyrimidines which would give the proper absorption spectra. Direct hydrolysis of the acid cleavage product gave an hydroxy sulfonic acid with the loss of a molecule of ammonia.

$$
\begin{array}{c}
N\!=\!C\!-\!NH_2 \\
\mid \quad \mid \\
H_3C\!-\!C \quad C\!-\!CH_3 \\
\parallel \quad \parallel \\
N\!-\!CH
\end{array}
$$
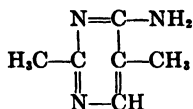
2,5-Dimethyl-4-aminopyrimidine

A comparison of the absorption spectra of isomeric pyrimidines of both the hydroxy and amino types led to the conclusion that the cleavage product was a 4-aminopyrimidine.

The final synthesis involved the coupling of the thiazole and pyrimidine structures, which was shown to be through the 5-methyoxy linkage of the pyrimidine and the nitrogen of the thiazole (Fig. 19c).

While vitamin B_1 does exhibit definite selective absorption, it is not usually determined chemically by this property, but rather by other spectral properties, as (1) fluorescence spectra, since it gives a brilliant fluorescence after oxidation with ferricyanide to yield a thiochrome (40), and (2) absorption spectra from dyes produced by coupling it with diazotized aromatic amines. Standard methods for the estimation of vitamin B_1 with a fluorimeter have been established (8, 65). The diazonium salts used in coupling to vitamin B_1 in the dye method of analysis have included those prepared from sulfanilic acid, p-aminoacetophenone, and 2,4-dichloroaniline (36).

2. *Vitamin B$_2$* *(Riboflavin)*

Vitamin B$_2$ is an essential part of several respiratory enzymes and is essential to certain nerve tissues. It is the pigment material in many enzymes. Its formula has been shown to be 6,7-dimethyl-9-(*d*-1'-ribityl) isalloxazine.

$$CH_2—CHOH—CHOH—CHOH—CH_2OH$$
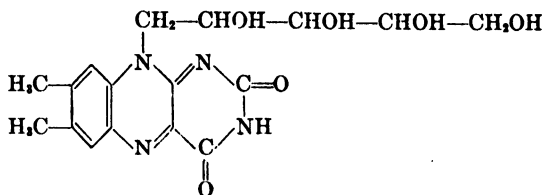
Vitamin B$_2$ (riboflavin)

Riboflavin is visibly colored yellow, with a green fluorescence. Its ultraviolet absorption spectrum shows even greater absorption than in the visual region, with a band at 265 mμ and about three times the extinction of the visual absorption band at 450 mμ (Fig. 20) (67). Riboflavin can
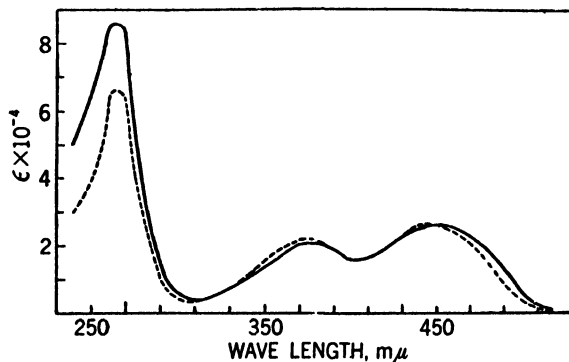
Fig. 20.—The absorption spectrum of vitamin B$_2$ (riboflavin), (———) and of alloxazine-adenine-nucleotide (- - - - -). (Warburg and Christian, 67).

be reduced to a leuco or colorless form which is easily oxidized back to its stable form and may account for some of its properties as a respiratory enzyme. The vitamin has also been identified as the pigment portion of a coenzyme derived from an amino acid oxidase. The enzyme is a protein–pigment complex, both parts of which are essential for the enzyme

property but whose absorption is characteristic of the resonating or riboflavin portion of the molecule (Fig. 20).

Kuhn (41) has described a spectrophotometric method for the determination of the amounts of flavins through the conversion of the riboflavin to lumiflavin and determination of the extinction at the absorption maxima of 470 mμ. From the known specific extinction of lumiflavin and riboflavin is it possible to convert the observed extinction values of the lumiflavin into per cent concentration of riboflavin. Fluorometric methods have been developed for the analysis of riboflavin (14, 26) and for mixtures of riboflavin and thiamine (36).

3. Nicotinic Acid (Vitamin P-P, Niacin)

Nicotinic acid (as well as nicotinamide) is the antipellagra factor. It tends to promote growth and maintain normal skin functions. The aromatic character of their formulas indicates that the compounds will have a resonating structure and exhibit selective absorption.
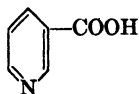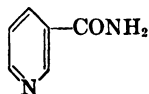
Nicotinic acid   Nicotinamide

The absorption spectra of nicotinic acid and its amide (40) are similar and, as would be expected from the known absorption spectrum of other pyridine derivatives, have a rather low extinction ($\epsilon = 3 \times 10^3$) for the band in the region of about 260 mμ. There is a band with a higher extinction ($\epsilon = 7 \times 10^3$) at about 215 mμ, but the position of the latter in the extreme ultraviolet and the usual general absorption of natural products near this region preclude the use of absorption spectra methods for the analysis and identification of this vitamin. Its marked stability to many reagents, however, permits the use of strong reagents in its separation and identification. Nicotinic acid and its derivatives are associated with certain coenzymes, and may impart absorption to these coenzymes or complicate absorption due to other resonating parts of the coenzyme.

Nicotinic acid can be determined spectrophotometrically by the use of the König reaction (pyridine derivative plus cyanogen bromide plus an aromatic amine), which yields a yellow color and which is best determined by the use of photoelectric colorimeters set to measure the transmission at the extinction maximum. Aniline has been used as the aromatic amine in this reaction (47), although recent work would indicate that metol which

gives a condensation product with its absorption band maximum at 300 mμ, is under some conditions a more satisfactory reagent than aniline (10). Other methods for the colorimetric estimation of nicotinic acid or its amide have been suggested, such as heating the amide with 2,4-dinitro-1-chlorobenzene and cleaving the salt formed with alkalies to yield an orange-colored derivative of glutaconic aldehyde (34).

4. Vitamin B₆ (Pyridoxine)

Vitamin B_6, or pyridoxine, is an antidermatitis factor, and is essential for the utilization of unsaturated fatty acids. A study of the absorption spectra of this vitamin and its related known compounds led to the assignment to it of the structure of 2-methyl-3-hydroxy-4,5-di(hydroxymethyl)-pyridine.
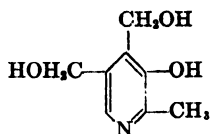
Vitamin B₆ (pyridoxine)
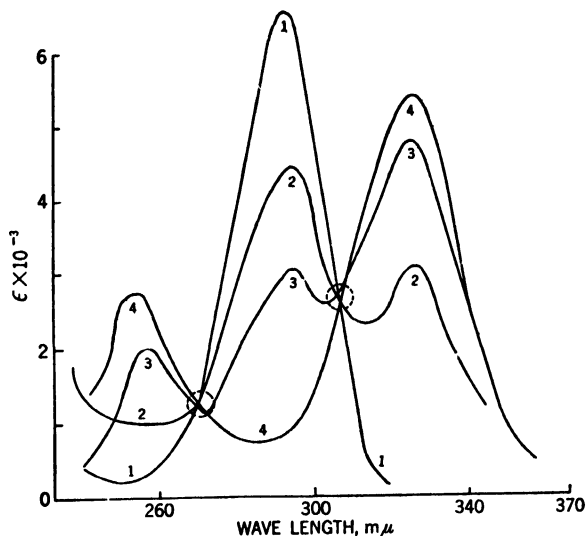
Fig. 21.—Influence of pH change on the absorption spectra of vitamin B₆ (pyrodoxin): (1) pH 2, (2) pH 4, (3) pH 5, and (4) pH 7. (Folkers, Stiller, et al., 20.)

The presence of the 3-hydroxy group gives the compound an aromatic phenolic type of absorption and leads to the tautomeric structures which are evidenced in the change of absorption with pH values. The marked differences in the absorption spectra of the 2-, 3-, and 4-hydroxy pyridines leave no doubt as to the location of the hydroxyl group in the vitamin when these compounds are examined spectrographically. It should be noted that, as in the case of indicators and other reactions involving a change of the resonator structure within a molecule to another resonator form, there are certain isosbestic points where the extinction remains constant at all intermediate values between the two forms. These points are circled in Figure 21.

Pyridoxine, like riboflavin, can couple with diazotized aromatic amines to produce dyes whose extinction at their absorption spectra maxima can be used to estimate the concentration and identity of the vitamin (61). Other color tests have been used for the estimation of pyridoxine, such as the reaction (58) with 2,6-dichloroquinonechlorimide to produce a blue color (absorption maxima at 660 mμ).

5. Inositol and p-Aminobenzoic Acid

These two compounds have been associated as members of the "B" family. Inositol as a saturated cyclic hexahydroxy alcohol exhibits no absorption. p-Aminobenzoic acid, shows a distinct and characteristic absorption and, through its ability to be diazotized and coupled to phenols or aromatic amines, gives additional resonating compounds which permit its exact estimation and identification.

X. Enzymes and Coenzymes

Enzymes are usually considered to be highly complex substances containing a protein coupled to some coenzyme. The coenzyme portion is often a resonating or pigment component with a definite and characteristic absorption which can be used to identify and aid in the isolation of the active constituent.

The coenzyme known as cozymase has been shown to have the structure given in the accompanying formula (15). It will be noted that there are pyridine and pyrimidine resonators. From the studies of Karrer (33) it would appear that the pyridine structure is capable of reduction to a leuco form, which might explain its property as an oxygen-transporting agent. In place of the nicotinic acid, a riboflavin nucleus may be similarly attached to produce a yellow coenzyme. The spectral effects shown by this

Nicotinamide

Adenine

Ribose

Ribose

Cozymase I
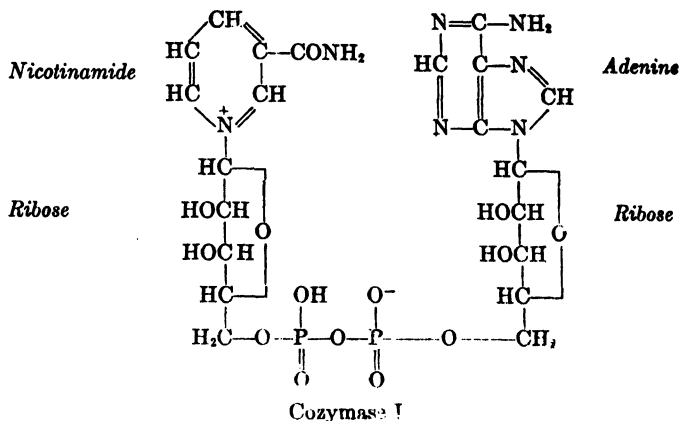
latter coenzyme will be closely related to those shown by riboflavin and the pyrimidines, since they are coupled through nonresonating linkages.

An amino acid oxidase has been shown by Warburg (66) to have a coenzyme structure of alloxazine-adenine-dinucleotide. The absorption spectra (43) of this coenzyme (Fig. 20) show that the principal absorption

Alloxazine

Adenine

Ribose

Ribose

Structure of d-amino acid oxidase
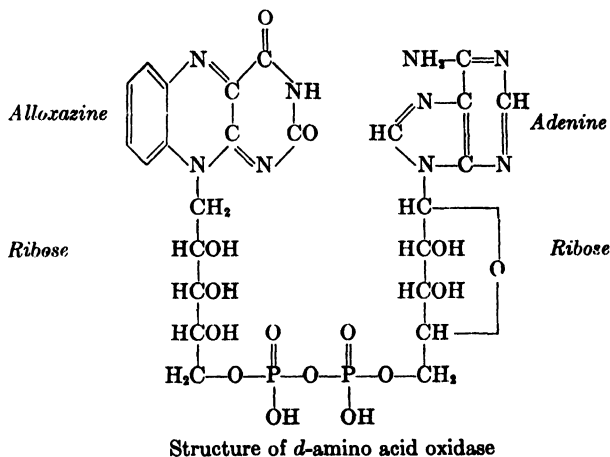
is due to the alloxazine portion (riboflavin or vitamin B_2) and that only in the extreme ultraviolet does there begin to appear additional absorption which would be characteristic of the purine absorption (adenine has an absorption maximum at 260 mμ. (See Figure 25, page 308).

Many of the porphyrins are associated as integral parts of enzymes, an

example of which is cytochrome c. This latter compound is one of several pigments (cytochrome a, b, and c) which form part of the oxidase. The complex structure proposed for cytochrome c (62) includes protein, carbo-
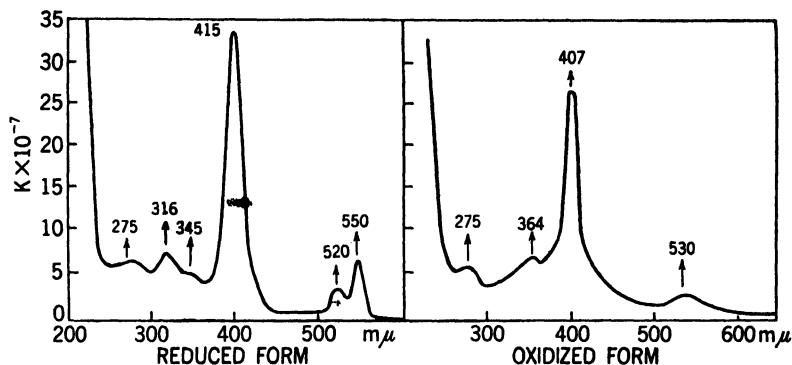
Fig. 22.—Absorption spectrum of cytochrome c. (Theorell, 62.)
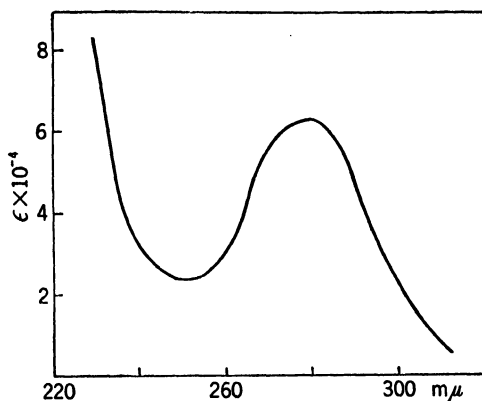
Fig. 23.—Absorption spectrum of trypsin. (Verbrugge, 63.)

hydrate, and porphyrin portions, of which the latter is responsible for the characteristic absorption bands and evidently for the oxygen transfer, in view of the marked changes in its absorption bands in the reduced and oxidized forms (Fig. 22).

The differentiation between cytochromes a, b, and c depends on the

observed absorption spectra and the location of the rather sharp and well-defined bands.

The application of absorption spectra methods to the general field of enzymes, coenzymes, nucleoproteins, and porphyrin compounds has been of great importance and has led to the rapid identification and separation of these materials (Fig. 23). It is not possible to consider these applications within this limited survey. Additional data on the methods are to be found in the work of Warburg and others and in recent reviews in this series by Agner (1) and by Mirsky (49).

XI.   Proteins, Purines, and Pyrimidines

These compounds, while not in themselves considered as vitamins or related compounds, are often integral parts of such natural substances
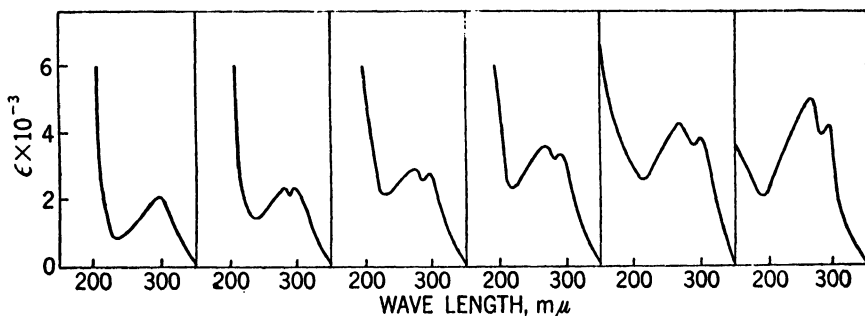
Fig. 24.—Absorption spectra of mixtures of tyrosine and tryptophane. Curves represent, from left to right, 100%, 80%, 60%, 40%, 20%, and 0% tyrosine where tyrosine plus tryptophane equals 100%. Curves observed in 0.1 N NaOH. (Holiday, 29.)

for which the observed absorption spectrum is often the result of these constituent parts. The simple amino acids and their combinations to form proteins do not produce selective absorption. However, the presence in the amino acid of resonator groups such as the benzene or pyrrolidine derivatives will cause absorption. It is thus possible to differentiate certain amino acids from others. In some cases, two amino acids, each with resonator groups, can be distinguished and their relative amounts determined through a spectroscopic examination of the mixture. This has been carried out for mixtures of tyrosine and trytophane by Holiday (29) (Fig. 24).

The absorption spectra of many of the amino acids have been determined in simple mixtures. If only one resonating form is known to be present, a spectroscopic estimation can be made of the resonating constituent without its separation from the other constituents. (Figure 6, page 277).
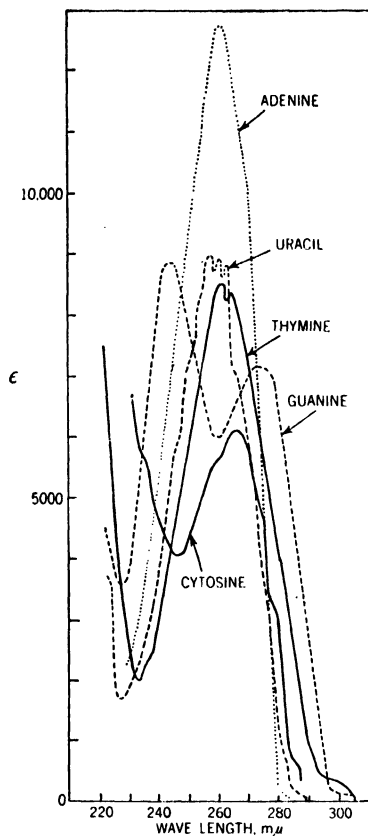
Fig. 25.—Absorption spectra of some naturally occurring pyrimidines. (Heyroth and Loofbourow, 24.)

The purines and pyrimidines are characterized by the resonating structures indicated in their formulas (24, 44), and both show selective absorption. The variation of the absorption of these compounds with pH conditions, due to their tautomeric natures, provides an additional means of
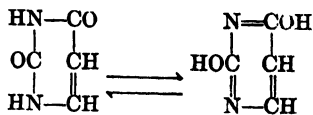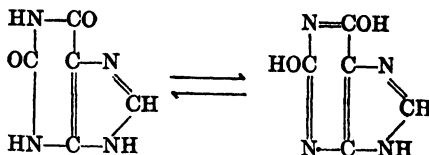
Pyrimidine   Purine
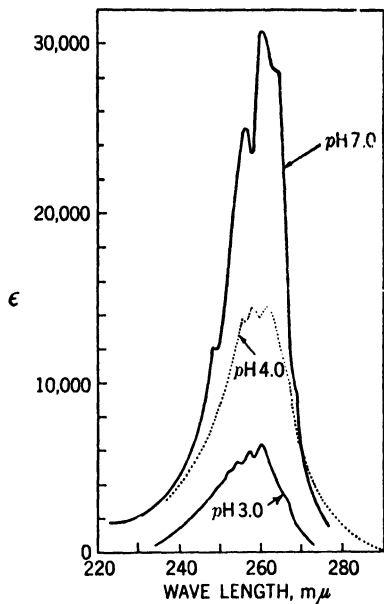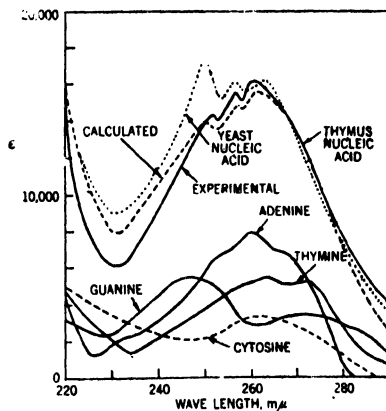
a   b

Fig. 26.—(a) Absorption spectra of barbituric acid in varying *p*H solution. (Loofbourow, 44.)

(b) Absorption spectra of thymus nucleic acid and the sum of its purine and pyrimidine constituents. (Loofbourow, 43; Loofbourow and Stimson, 44.)

spectral identification.   Because they are essential parts of certain vitamins hormones, and enzymes, the determination of the absorption spectra of these cyclic nitrogen compounds has aided considerably in the prediction of the structure of the complex natural products (22, 24) (Figs. 25 and 26).

Bibliography

1. Agner, K., *Advances in Enzymology*, Vol. III.   Interscience, New York, 1943, p. 137.

2. Bath, J. W., and Ellis, J. W., *J. Phys. Chem.*, **45**, 204 (1941).
3. Brode, W. R., *J. Am. Chem. Soc.*, **46**, 581 (1924).
4. Brode, W. R., *Chemical Spectroscopy*. 2nd ed., Wiley, New York, 1943.
5. Brode, W. R., and Magill, M. A., *J. Biol. Chem.*, **92**, 87 (1931).
6. Brode, W. R., and Magill, M. A., *Ohio J. Sci.*, **33**, 127 (1933).
7. Carr, F. H., and Price, E. A., *Biochem. J.*, **31**, 497 (1926).
8. Conner, R. T., and Straub, G. J., *Ind. Eng. Chem., Anal. Ed.*, **13**, 385 (1941).
9. Coy, N. H., Sassaman, H. L., and Black, A., *ibid.*, **15**, 441 (1943).
10. Dann, W. J., and Handler, P., *J. Biol. Chem.*, **140**, 201 (1941).
11. Dirscherl, W., and Hanusch, F., *Z. physiol. Chem.*, **233**, 13 (1935).
12. Doisy, E. A., *et al.*, *J. Am. Chem. Soc.*, **61**, 1295, 1928, 2558 (1939).
13. Ellinger, F. *Tabulae Biologicae.* Vol. 12, Junk, The Hague, 1937, p. 291.
14. Emmett, A. D., *Ind. Eng. Chem., Anal. Ed.*, **13**, 219 (1941).
15. Euler, H. von, and Schlenk, F., *Z. physiol. Chem.*, **246**, 64 (1936).
16. Ewing, D. T., Kingsley, G. V., Brown, R. A., and Emmett, A. D., *Ind. Eng. Chem., Anal. Ed.*, **15**, 301 (1943).
17. Ewing, D. T., Tomkins, F. S., and Kamm, O., *J. Biol. Chem.*, **147**, 233 (1943).
18. Ewing, D. T., Vandenbelt, J. M., and Kamm, O., *ibid.*, **131**, 352 (1939).
19. Fieser, L. F., *et al.*, *J. Am. Chem. Soc.*, **61**, 1925, 2206, 2559 (1939).
20. Folkers, K., Stiller, E. T., *et al.*, *ibid.*, **61**, 1237, 1242, 1245 (1939).
21. Fraenkel-Conrat, H., *Ann. Rev. Biochem.*, **12**, 273 (1943).
22. Gulland, J. M., *J. Chem. Soc.*, **1938**, 1722.
23. Heyroth, F. F., Ellis, C., and Wells, A. A., *The Chemical Action of Ultraviolet Rays.* Rev. ed., Reinhold, New York, 1941.
24. Heyroth, F. F., and Loofbourow, J. R., *J. Am. Chem. Soc.*, **56**, 1728 (1934).
25. Hochberg, M., Melnick, D., and Oser, B. L., *Ind. Eng. Chem., Anal. Ed.*, **15**, 182 (1943).
26. Hodson, A. Z., and Norris, L. C., *J. Biol. Chem.*, **131**, 621 (1939).
27. Hogness, T. R., *et al.*, *ibid.*, **120**, 239 (1937).
28. Hogness, T. R., and Potter, V. R., *Ann. Rev. Biochem.*, **10**, 509 (1941).
29. Holiday, E. R., *Biochem. J.*, **24**, 619 (1930); **30**, 1795 (1936); **32**, 1166 (1938).
30. Holiday, E. R., *Brit. J. Exptl. Path.*, **23**, 103 (1942).
31. Johnson, S. W., *Biochem. J.*, **30**, 1430 (1936).
32. Karrer, P., *et al.*, *Helv. Chim. Acta*, **22**, 310 (1939).
33. Karrer, P., and Benz, F., *ibid.*, **19**, 1028 (1936).
34. Karrer, P., and Keller, H., *ibid.*, **21**, 463 (1938).
35. Kass, J. P., Miller, E. S., and Burr, G. O., *Abstracts of Papers, 99th Meeting Am. Chem. Soc.*, Cincinnati, Ohio (1940).
36. Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, **32**, 1516 (1938).
37. Knorr, H. V., and Albers, V. M., *Rev. Sci. Instruments*, **8**, 183 (1937).
38. Koenig, R. A., Schiefelbusch, T. L., and Johnson, C. R., *Ind. Eng. Chem., Anal. Ed.*, **15**, 181 (1943).
39. Kuhn, R., Hausser, K. W., and Seitz, G., *Z. physik. Chem.*, **B29**, 391 (1935).
40. Kuhn, R., and Vetter, H., *Ber.*, **68**, 2374 (1935).
41. Kuhn, R., Wagner-Jauregg, T., and Kaltschmitt, H., *ibid.*, **67**, 1452 (1934).
42. Lewis, G. N., and Calvin, M., *Chem. Revs.*, **25**, 273 (1939).
43. Loofbourow, J. R., *Rev. Modern Phys.*, **12**, 267 (1940).
44. Loofbourow, J. R., and Stimson, M. M., *J. Chem. Soc.*, **1940**, 1275.

45. Lopez, J. M., *Anales soc. cient. argentine*, 101, 133 (1926). *Tables annuelles de constantes et données numériques*, 8, 1313 (1928).

46. Marenzi, A. D., and Vilallanga, F., *Rev. soc. argentina biol.*, 17, 232 (1941).

47. Melnick, D., and Field, H., *J. Biol. Chem.*, 134, 1 (1940).

48. Miller, E. S., *Quantitative Biological Spectroscopy*. Burgess, Minneapolis, 1939. Rev. ed., 1940.

49. Mirsky, A. E., *Advances in Enzymology*, Vol. III. Interscience, New York, 1943, p. 1.

50. Mitchell, J. H., Kraybill, H. R., and Zscheile, F. R., *Ind. Eng. Chem., Anal. Ed.*, 15, 1 (1943).

51. Mohler, H., and Lohr, H., *Helv. Chim. Acta,* 21, 485 (1938).

52. Morton, R. A., *Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes*. 2nd ed., Hilger, London; Jarrell-Ash, Boston, 1942.

53. Morton, R. A., *Ann. Rev. Biochem.*, 11, 365 (1942).

54. Morton, R. A., Heilbron, I. M., and Gillam, A. E., *Biochem. J.*, 25, 1352 (1931).

55. Mowry, D. T., Brode, W. R., and Brown, J. B., *J. Biol. Chem.*, 142, 679 (1942).

56. Niemann, C., *et al.*, *J. Am. Chem. Soc.*, 63, 1549, 2685 (1941).

57. Patterson, J. W., Brode, W. R., and Brown, J. B., *Abstracts of Doctorate Theses*. Ohio State Univ., Columbus, 1942.

58. Scudi, J. V., Koones, H. F., and Keresztesy, J. C., *Am. J. Physiol.*, 129, 459 (1940).

59. Sims, H. B., and Scott, D. A., *Trans. Roy. Soc. Can.*, 24, V, 117 (1930).

60. Smith, L. I., *Chem. Revs.*, 27, 287 (1940).

61. Swaminathan, M., *Nature*, 145, 780 (1940).

62. Theorell, H., *Biochem. Z.*, 285, 207 (1936).

63. Verbrugge, F., *J. Biol. Chem.*, 149, 405 (1943).

64. Vigneaud, V. du, *et al.*, *ibid.*, 145, 503 (1942); *Science*, 96, 455 (1942).

65. Wang, Y. L., and Harris, L. J., *Biochem. J.*, 33, 1356 (1939).

66. Warburg, O., and Christian, W., *Biochem. Z.*, 298, 150 (1938).

67. Warburg, O., and Christian, W., *ibid.*, 298, 164 (1938).

68. Webb, T. J., *et al.*, *J. Org. Chem.*, 4, 389 (1939).

69. Williams, R. R., Ruehle, A. E., *et al.*, *J. Am. Chem. Soc.*, 59, 526, 530, 1052 (1937).

AUTHOR INDEX*

A

Abderhalden, E., 75 (ref. 1), *95*
Abrams, R., 175, *197*
Abramson, H. A., 148 (ref. 1), *150*
Ackerman, H., 173 (ref. 151), *198*
Adams, M., 63 (ref. 1), 65–67, *72*
Adams, Mark H., 108, 109, 116, 118 (ref. 2), 120, 121, 126 (ref. 2), 127 (ref. 3), 128, *150*
Adler, E., 91 (ref. 2), *95*, 210, 245 (ref. 1), *250*
Agner, K., 307, *309*
Ågren, G., 89 (ref. 3), 93, *95*
Akasi, S., 80, *95*
Albaum, H. G., 212, 230 (ref. 167). *254*
Albers, V. M., 276, *310*
Alexander, A. E., *33*
Allcroft, R., 178 (ref. 1), *195*
Allen, F. W., *34*
Allen, T. H., 101 (ref. 4), 102 (ref. 6, 7), 106' *150*
Altenberg, E., 17 (ref. 141), *37*
Althausen, T. L., 263 (ref. 1), *265*
Altshuler, C. H., 190 (ref. 138), 217 (ref. 184), *198*, *255*
Ames, S., 147, *151*
Anderson, E., 258 (ref. 43), 261 (ref. 42), 263, *267*
Anderson, E. M., 263 (ref. 1), *265*
Anderson, R. L., 23, *34*
Anderson, T. F., 4 (ref. 187), 5 (ref. 187), *38*
Andersson, K. J. I., 3 (ref. 131), *36*
Andervont, H. B., 242 (ref. 85), 244 (ref. 85), *252*
Annau, E., 237 (ref. 3), *250*
Anson, M. L., *33*, 149, *151*
Appel, H., 48 (ref. 10), *73*
Appenzeller, R., 84 (ref. 43), *96*
Ark, P. A., 157, *195*
Arnim, K. von, 75 (ref. 32), *96*
Arnow, L. E., 101, *150*
Arrick, M. S., *35*
Astbury, W. T., 2 (ref. 5, 6), 6, 13 (ref. 5), *33*, *34*, 217 (ref. 9), *250*
Atkin, L., 212, *250*
Auerswald, H., 155 (ref. 3), *195*
Axelrod, A. E., 236, 239, *250*, *255*
Ayyar, C. V. R., 102 (ref. 54), *151*
Azarkh, R. M., 86 (ref. 7), 87 (ref. 6), 90 (ref. 6), 91 (ref. 6), *95*

B

Bach, A., 102, 103, *150*
Bach, S. J., 260, *266*
Bailey, K., 217 (ref. 8, 9), 220, 221, *250*
Baker, D. L., 143, *150*
Baker, M. D., 206 (ref. 185, 186), *255*
Baker, Z., 174, 184 (ref. 4), *195*, 203 (ref. 68), 204 (ref. 67–69), 206 (ref. 10), 240 (ref. 69), *250*, *252*, 259 (ref. 17), *266*
Ball, E. G., 119 (ref. 10), *150*
Balls, A. K., 160, 193 (ref. 5), 194 (ref. 5), *195*
Baltzer, F., 26, *34*
Banga, I., 217 (ref. 12), **237** (ref. 11), *250*
Barigozzi, C., 15 (ref. 8), *34*
Barker, S. B., 257, 259, *266*, *267*
Barkhash, A. P., 211 (ref. 76), *252*
Barnard, J. E., 8 (ref. 9), *34*
Barnes, L. H., 170 (ref. 118), 173 (ref. 118), *198*
Barrón, E. S. G., 204, 237 (ref. 15), 238, 249, *250*, *253*
Bass, L. W., *36*
Bassett, A. M., 204 (ref. 53), 205, 240 (ref. 53), *251*, 261 (ref. 54), *267*
Bath, J. W., 286 (ref. 2), *310*
Bauch, M., 206 (ref. 138), *253*
Baumann, C. A., 228 (ref. 119), *253*, 259, *267*
Bayne-Jones, S., 202 (ref. 16), 203 (ref. 16), *250*
Beadle, G. W., 19 (ref. 193), 21, 23, 30, *34*, *38*
Beale, G. H., *34*
Bean, C. W., 178 (ref. 6), *195*
Beard, D., 229 (ref. 194), 230 (ref. 17), *250*, *255*
Beard, J. W., 229 (ref. 194), 230 (ref. 17), *250*, *255*
Becker, E., 17 (ref. 151), 23 (ref. 31, 151), *34*, *37*
Behm, R. C., 137–141, *150*
Behrens, Martin, 12, *34*
Belitser, V. A., 215, 221, *250*
Bell, F. O., 2 (ref. 5, 6), 6, 13 (ref. 5), *33*, *34*
Bellows, J. G., 177, 180 (ref. 7), *195*
Bendall, J. R., 263, *266*
Benoy, M. P., 204 (ref. 19, 69, 72), 240 (ref. 69), *250*, *252*
Benz, F., 304 (ref. 33), *310*
Berenblum, I., 228 (ref. 20, 21), *251*

---

* Italic numerals refer to the bibliographies of the different papers.

313

Bergmann, Max, 3, 14 (ref. 14), *34*, 164, *195*, *196*, *198*
Bernal, J. D., 6 (ref. 17), 14, *34*
Bernheim, F., 245 (ref. 22), *251*
Berrill, N. J., 203, 229, *251*
Bertrand, G., *34*, 100, 102 (ref. 14), 115, *150*
Besta, B., 155 (ref. 9), *195*
Bevan, E. J., 112 (ref. 25), *150*
Bhagvat, K., 101, *150*
Biechele, O., 12 (ref. 22), *34*
Black, A., 275 (ref. 9), *310*
Blair, J. E., 173 (ref. 10), *195*
Blair, M. G., 69
Block, R. J., *34*
Blum, H. F., 228, *251*
Boas, F., 12 (ref. 22), *34*
Bodine, J. H., 101, 102, 106, *150*
Boivin, A., 174 (ref. 11), *195*
Bollman, J. L., 220, 224, *252*
Bolomey, R. A., *34*
Bordley, J. E., 177, 180 (ref. 12), *195*
Bordner, C. A., 112, 134, 135, 137, *150*
Boswell, J. G., 127 (ref. 18), 142, 143, *150*
Bougault, M. J., 101, *150*
Bourdillon, J., 8, *34*
Bourquelot, E., 45 (ref. 2), *72*, 100, *150*
Boyer, P. D., 214 (ref. 25, 26), 223 (ref. 26), *251*
Boyland, E., 92 (ref. 17), *95*, 207, 245 (ref. 28, 29), 248 (ref. 27), *251*
Boyland, M. E., 207 (ref. 28, 29), 245 (ref. 28, 29), 248 (ref. 27), *251*
Brachet, J., 231, *251*
Brand, E., *34*
Braunshtein, A. E., 76, 82, 83, 85, 86, 87 (ref. 5, 6), 89 (ref. 5, 9), 90 (ref. 5, 6), 91 (ref. 6), 92, 93, *95*
Breusch, F. L., 89 (ref. 12), 90 (ref. 12), *95*, 210, *251*
Brewer, C. M., 174 (ref. 13), *195*
Brewer, S. D., 81, 84 (ref. 13), *95*
Brieger, G., 46 (ref. 11), *73*
Brinkhous, K. M., *34*
Britton, S. W., 264, *266*
Brode, W. R., 270 (ref. 4), 272 (ref. 4), 273, 277, 280 (ref. 55, 57), 281 (ref. 55, 57), 282 (ref. 5), 284, *310*, *311*
Brömel, H., 214 (ref. 151), *222* (ref. 151), *254*
Brown, B. H., 26 (ref. 109), *36*
Brown, J. B., 280 (ref. 55, 57), 281 (ref. 55, 57), *311*
Brown, R. A., 292 (ref. 16), *310*
Browne, J. S. L., 265, *266*
Bruce, H. M., 263 (ref. 4), *266*
Bryan, C. S., 178 (ref. 14, 15, 69), *195*, *196*
Buchmann, W., *34*
Bull, H. B., 129 (ref. 21), *150*
Bumbacher, H., 144, *151*
Bunding, I. M., 16, *34*
Bureau, V., 16 (ref. 30), *34*

Burian, R., 13, *34*
Burk, D., 203, 204 (ref. 32–34), 206 (ref. 32), 213, *251*
Burk, R. E., 44 (ref. 3), *72*
Burr, G. O., 280 (ref. 35), *310*
Burt, C. P., 59 (ref. 12), *73*
Butenandt, A., 23, *34*
Bychov, S. M., 87, 92, 93, *95*

C

Cahill, G. F., *34*
Califano, L., 102 (ref. 22), 133, 142, *150*
Calvin, M., *34*, 271 (ref. 42), *310*
Cambier, M. J., 176 (ref. 155), *199*
Canneyt, J. van, 155, *195*
Canzanelli, A., 261, *266*
Carandante, G., 92, *95*
Carpenter, C. M., 173 (ref. 151), *198*
Carr, F. H., 282 (ref. 7), *310*
Caspersson, T., 8 (ref. 33), 12 (ref. 177), 13 (ref. 33), 14, *34*, *38*, 231, *251*
Castle, W. E., 25, 26, 30, 31, *34*
Catchside, D. D., *36*
Cattaneo, C., 156 (ref. 32), 157, 160 (ref. 32), 170 (ref. 32), 176 (ref. 32), 184 (ref. 32), *196*
Cavier, R., 174 (ref. 159), *199*
Cedrangolo, F., 92, *95*
Chaffee, E., 170 (ref. 63), 172 (ref. 63), *196*
Chaikoff, I. L., 189 (ref. 142), *198*, 258, 261 (ref. 42), 264, *267*
Chambers, L. A., 8, 12, *34*
Chambers, S. O., 156 (ref. 17), *195*
Chambers, W. H., 257, *266*
Chapman, A., 261 (ref. 7), *266*
Chen, T. T., 119 (ref. 10), *150*
Cheng, C., 62, *74*
Cherbuliez, E., 75 (ref. 16), *95*
Chibnall, A. C., 92 (ref. 17), *95*
Chodat, R., 100, 102, 103, 115 (ref. 24), *150*
Chou, C.-Y., 164 (ref. 147), *198*
Christensen, H. N., 162 (ref. 19), 163 (ref. 19), 164, 166 (ref. 18), 167 (ref. 19), 176 (ref. 19), 179 (ref. 19), *195*
Christian, W., 187 (ref. 164), 190 (ref. 164), *199*, 211 (ref. 221, 222), 214 (ref. 224), 222, 226, 232 (ref. 225), 240, 247, *256*, 301, 305 (ref. 66), *311*
Churney, L., *34*
Clapp, M. P., 179 (ref. 20), *195*
Clark, G. F., 178 (ref. 14, 69), *195*, *196*
Clark, William G., 263, *266*
Claude, A., *34*, 203 (ref. 34), 204 (ref. 34), 211 (ref. 48, 52), 229 (ref. 47, 50), 230–233, *251*
Clifton, C. E., 187 (ref. 120), 191 (ref. 21, 119, 120), *195*, *198*
Coburn, A. F., 170 (ref. 33), 173 (ref. 33), 174 (ref. 33), 184, 185 (ref. 36), *196*
Code, C. F., 227 (ref. 196), *255*

SUBJECT INDEX

A

Absorption spectra, 270–309. See also *Chromophoric changes* and under specific compounds.
chromophores, 272–274
combination of resonators, 273–276
frequency shifts, 273–276
fundamentals, 271–278
intensity shifts, 272–276
methods of recording, 270–271
resonance, 272–278
and structure of molecule, 270–277
Adenosine diphosphate, 208, 222, 223, 227
Adenosinetriphosphatase, and myosin, 216, 217, 218, 220, 238
in normal and cancer tissues, 243–244
Adenosine triphosphate. See also *Growth control.*
breakdown and glycolysis, 219, 225
and growth control, 218–223, 225, 226
and pharmacological control, 219, 224–225
as energy reservoir, 218
in glycogen synthesis, 216, 218, 222
methods of assay, 238
priming reactions, phosphorylation of glucose, 213–214
phosphorylation of glycerol, 213
and staphylococci, 186
synthesis, 214–216
utilization, 216–218
Adenylic acid in normal and cancer cells, 208, 210, 214, 216, 222, 223, 226, 227, 238
ADP. See *Adenosine diphosphate.*
Adrenaline, absorption spectrum, 285, 286
epinephrine and glycogen metabolism, 262
Aglycon group (in glycosides), alkyl group, 53–59
aryl group, 59–61
in glucosides, 53–61, 62–64
in glycosides, 42, 43, 45
structure, chain length and adsorption on substrate, 54–58
Almond emulsin, enzyme constituents, 46–48
β-glucosidase from. See *β-Glucosidase.*
hydrolyzing action on different glycosides, 47, 48
purification of enzymes from, 45–46
Amino acids. See also *Proteins, Polypeptides.*

absorption spectra, 277, 279
deamination of α-acids, 75–76
and keto acids, interaction, 77–79
optical activity, change during transamination, 77–78
optical rotation and biological activity, 9
during transamination, 77–78
ratio, in insulin, 3
in tobacco mosaic virus, 7
steady state in protein molecule, 11
tyrosine, 100, 115, 144–145
"unnatural" or *d*-amino acids in Tyrocidine and Gramicidin, 154, 163, 164, 165, 166, 168, 169, 183
p-Aminobenzoic acid, absorption spectrum, 285, 304
Amino acid oxidase, absorption spectrum, 301, 305
Aminopherase, 86
Androsterone, absorption spectrum, 288, 289
Antibacterial agents. See *Gramicidin, Tyrocidine, Tyrothricin.*
Antibiotic effects of aerobic, spore-forming bacteria, 154
Ascorbic acid. See *Vitamin C.*
ATP. See *Adenosine triphosphate.*
Autosynthetic molecules, 4–8
Autosynthetic process, 8–11, 19

B

Bacillus brevis, bactericidal substances from, biological properties, 169–182
mode of action 182–194
purification and chemistry, 156, 157–169
Bacteria. See also under specific organism.
aerobic, spore-forming. See also *bacillus brevis.*
bacteriolytic effects of filtrates, 153–157
Gram-negative, 154, 156, 170, 171, 172, 173, 174, 175
Gram-positive, 154, 156, 158, 170, 171, 172, 173, 174, 185
Bactericidal action, 154, 155, 169, 185
Bacteriolysis, 169, 192. See also *Tyrocidine.*
Bacteriostatic action of gramicidin, 169, 185, 188, 189
Bergmann ratios, 2, 3, 10
Biotin, in cancer and normal tissues, 240, 248
derivatives, absorption spectra, 278–279

325

CUMULATIVE INDEX OF VOLUMES I–IV

A. Author Index

B. Subject Index

.

.

DATE OF ISSUE

For Reference
Only.